Toxicology of the male reproductive tract; associations with smoking and antioxidants

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Toxicology of the Male Reproductive Tract; Associations with Smoking and Antioxidants

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Abstract

The integrity of the male reproductive system is essential in order to ensure the future well-being of a species. This study aimed to determine whether cigarette smoking and/or low antioxidant levels are associated with damage to human spermatozoa that may result in offspring pathology and reduced male fertility.

Using the sperm chromatin structure assay and the terminal deoxynucleotidyl transferase assay, it was determined that smokers' sperm were significantly more sensitive to acid induced denaturation and possessed higher levels of DNA strand breaks than non-smokers' sperm. In addition, sperm from donors who smoked possessed more YY1 aneuploid sperm compared to non-smokers. These differences may predispose offspring of paternal smokers to greater risk of disease and malformations. The total antioxidant activity of seminal plasma from smokers was also significantly lower than that of non-smokers.

Sperm preparation techniques, such as centrifugation, commonly used prior to in vitro fertilisation and scientific studies, are associated with spermatozoal damage. It was demonstrated that the induction by hydrogen peroxide of spermatozoal DNA strand breaks and lipid peroxidation was reduced by the presence of seminal plasma. The results indicate that maintaining sperm in seminal plasma might decrease the potential for cellular damage to arise and allow for an improvement in semen quality used for artificial insemination and scientific research.

The human epididymis provides an optimal environment for the storage and maturation of spermatozoa. However, extra-epididymal spermatozoa account for approximately a third of all sperm cells found in the normal human ejaculate. Data from the study determined that sperm living outside of the epididymis express an abnormal chromatin structure, by comparing sperm from patients after vasectomy to normozoospermic donors. It was also demonstrated that the epididymis possesses region-specific antioxidant activity, which may protect sperm from oxidative attack during storage at this site.

Supercritical fluid technology allowed the development of a novel method to extract cotinine, a nicotine metabolite, and caffeine, from semen. HPLC and GC-MS were used to identify the compounds. GC-MS also allowed the identification of several

hydrocarbons in seminal fluid, including an aromatic hydrocarbon, that may have toxicological implications. Coenzyme Q_{10}, an antioxidant and component of the respiratory chain, was also measured in spermatozoa and seminal plasma. Spermatozoal levels of coenzyme Q_{10} correlated with sperm count, motility and normal morphology, but was found at equal levels in samples obtained from smokers and non-smokers.

It is hoped that the data obtained in the study will enable advancement in the treatment of infertility, and provide information on potential causes of offspring disease and abnormalities.

Contents

Abbreviations

ABTS	2'2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)
C18	octadecyl carbon chain
CAT	catalase
CNS	central nervous system
CoQ_{10}	coenzyme Q_{10}
DAPI	4'6-diamidino-2-phenylindole
EC-SOD	extra-cellular superoxide dismutase
FISH	fluorescence in situ hybridisation
GC-MS	gas chromatography-mass spectrometry
GPX	glutathione peroxidase
GRD	glutathione reductase
GSH	glutathione
GSSG	glutathione disulphide
H_20_2	hydrogen peroxide
HPLC	high performance liquid chromatography
MDA	malondialdehyde
Mn-SOD	manganese-superoxide dismutase
MetMb	metmyoglobin
NADH	nicotine adenine dinucleotide
NNK	4-(methylnitrosamino-)-1-(3-pyridyl)-1-butanone
NNN	N-nitrosonornicotine
PAH	polycyclic aromatic hydrocarbons
PBD	phosphate buffered detergent
PBS	phosphate buffered saline
SCSA	sperm chromatin structure assay
SFE	supercritical fluid extraction
SOD	superoxide dismutase
SPE	solid phase extraction
SSC	sodium citrate, sodium chloride
TBARS	thiobarbituric acid reactive substances
TdT	terminal deoxynucleotidyl transferase
TdTA	terminal deoxynucleotidyl transferase assay
TSNA	tobacco-specific nitrosamines
Cu,ZN-SOD	copper-zinc-superoxide dismutase

1. Introduction

The reproductive system enables the perpetuation of a species. The survival of an organism is dependent on the integrity of this system which is essential in order to ensure the flawless flow of genetic material from generation to generation. It is estimated that approximately twenty percent of couples are involuntary sterile and around fifteen percent of recognised pregnancies spontaneously abort (Thomas 1996). The impact of chemicals on the reproductive system is, therefore, an important area of research due to the adverse effects of many such chemicals, causing, for example, reduced fertility and genetic damage to the gametes.

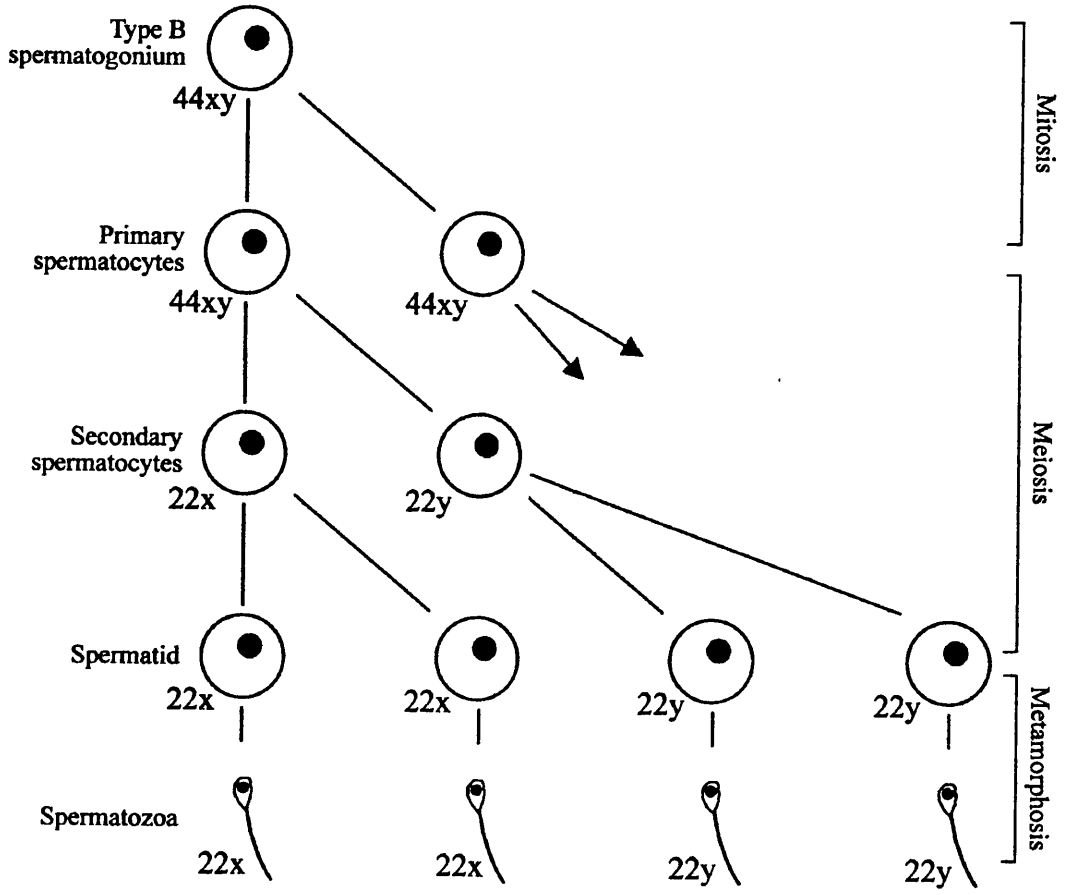
1.1 Physiology of the Male Reproductive Tract

The male gonads possess two functions; firstly, the synthesis and secretion of sex hormones, predominantly testosterone and dihydrotestosterone, and secondly, gametogenesis, or the production of germ cells. Both functions are dependent on the secretions of the anterior pituitary gland, namely follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The secretion of FSH and LH is stimulated by the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus. FSH stimulates spermatogenesis by binding to a receptor located on the Sertoli cells. This causes the Sertoli cells to secrete a host of hormones and proteins that stimulate germ cell production. LH increases the rate of testicular steroidogenesis. The release of GnRH, FSH and LH is inhibited by hormonal products of the testes, such as testosterone; a process termed 'negative feedback'.

1.1.1 *Spermatogenesis: the Production of Sperm*

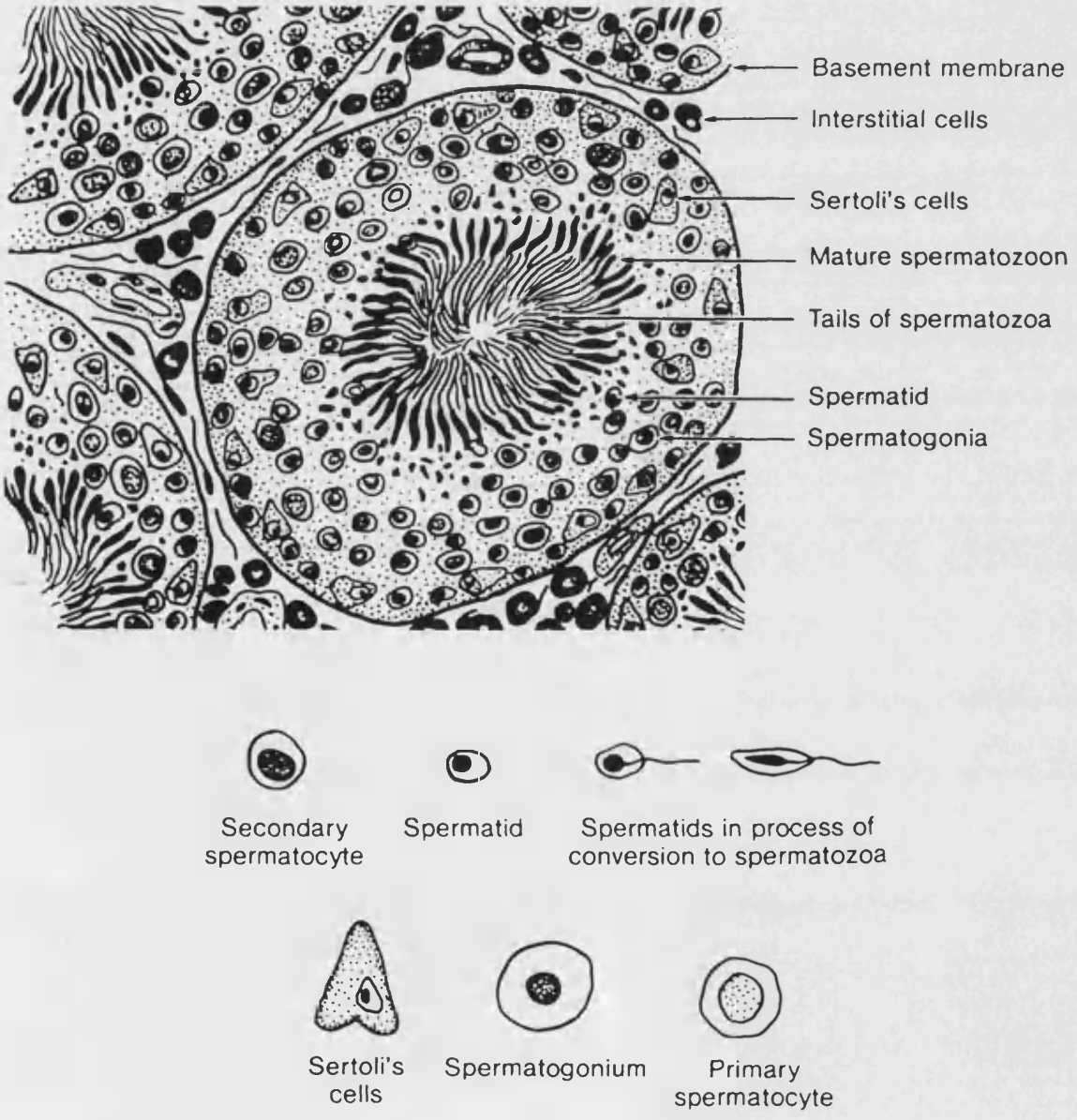
Spermatogenesis is the process whereby male germ cells, or spermatozoa, are produced within the germinal epithelia (Amann 1989). Each day, the germinal epithelia must produce millions of spermatozoa from primitive germ cells, the spermatogonia. Of the two major types of spermatogonia, type A generate more spermatogonia. Type B spermatogonia go on to become mature spermatozoa through a multi-step process, as depicted in fig. 1.1. Once the testes have fully matured upon puberty, the type B spermatogonia develop into primary spermatocytes, which divide through meiotic cell division to become secondary spermatocytes and then spermatids. Meiotic cell division results in a halving of the normal complement of chromosomes; from a diploid cell to a haploid cell. Spermatids complete their development into spermatozoa through extensive nuclear and cytoplasmic reorganisation (Bedford 1979; Balhorn 1989). The nucleus containing the cell's genetic material, condenses and becomes the sperm head. This process occurs as the nuclear histones are replaced by arginine/cysteine-rich protamines, which allows chromatin stabilisation through intra- and inter-protamine cross-linking via disulphide bridges. This produces a highly condensed sperm nucleus whose volume is only 1 : 100,000 that of an ovum, and which is more resistant to DNA cleavage and denaturation and in situ enzymatic degradation than a somatic cell. In addition, the two centrioles form the flagellum, and mitochondria migrate to a sheath situated nearby the flagellum. Both the mitochondria and flagellum enable sperm cell motility. Part of the Golgi apparatus also moves to the head of the cell into the acrosome region; a process required for penetration of the oocyte at fertilisation.

Fig. 1.1. The production of spermatozoa, or spermatogenesis. The numbers and letters next to each cell represents the chromosome compliment of the cell. For example, 44xy denotes a cell possessing 44 autosomes and one of each sex chromosomes (source: Thomas 1996).



The process of spermatogenesis occurs within the seminiferous tubules, which are represented in fig. 1.2. Found within the interstitium of the tubules are the Leydig cells, the primary site of male testosterone synthesis (Ewing 1992). Conversely, the germ cells and Sertoli cells are located within the membranous boundaries of the seminiferous tubules. The integrity of the Sertoli cells is essential for normal spermatogenesis (Thomas 1996). The Sertoli cells secrete several key hormones and proteins such as inhibin, which modulates pituitary FSH secretion, and androgen-binding protein that acts as a carrier of testosterone and dihydrotestosterone. In addition, the Sertoli cell junctions aid in the formation of the blood-testes barrier.

Fig. 1.2. A schematic cross-section of testicular seminiferous tubules (source: Thomas 1996).



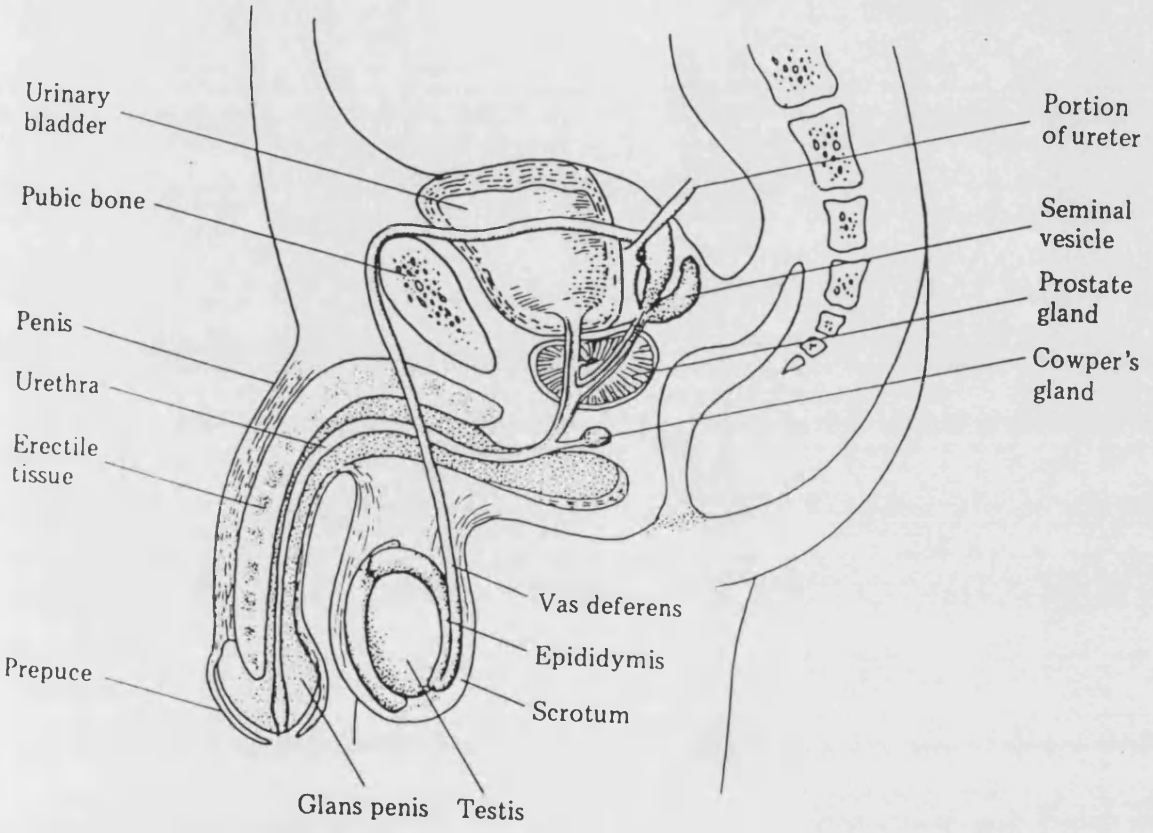
Following spermatogenesis, the sperm are expelled from the testes into the epididymis through the efferent ducts by way of the rete testes. Prior to ejaculation, several post-testicular secretions are added to the sperm, which when combined, produces seminal fluid or semen.

1.1.2 *Accessory Sex Organs*

A number of organs, known as the accessory sex organs (see fig. 1.3), contribute to the post-testicular processes. The seminal plasma transports the ejaculated sperm from the male to the female reproductive tract. The secretory organs, including the epididymides, prostate, seminal vesicles, bulbourethral (or Cowper's) glands, and urethral (or Littre's) glands, produce the seminal plasma. Abnormal function of any of the secretory organs may be reflected in an alteration of the characteristics of the seminal plasma (Thomas 1996).

The human ejaculate is produced sequentially (Thomas 1996). The first portion of the ejaculate, or pre-sperm fraction, is comprised of secretions produced by the urethral and Cowper's glands. The sperm-rich fraction then follows, and contains prostatic secretions and the spermatozoa. Finally, the seminal vesicle secretion is delivered, comprising the post-sperm portion. There is a considerable overlap between the pre-sperm, sperm-rich, and post-sperm fractions. It is, therefore, very difficult to obtain a sperm-free fraction consisting exclusively of seminal vesicular secretions for example.

Fig. 1.3. Anatomical arrangement of the male reproductive tract, including the accessory sex organs (source: Guttman and Hopkins, 1983).



Several biochemical markers exist for the accessory gland secretions (WHO 1992). The prostatic secretion in men and many other mammals is indicated by acid phosphatase, zinc and citric acid, whereas fructose is a marker for active seminal vesicle secretion. About two-thirds of the total human ejaculate is contributed by the seminal vesicles and the other third by the prostate.

The World Health Organisation (WHO) publishes criteria that should be met by a sample in order for it to be considered 'normozoospermic', or 'normal' (WHO 1992), which is shown in table 1.1. In order for a semen sample to be considered normozoospermic, the specimen should possess a minimal sperm cell concentration of 20 x 10^6/ml, a minimum forward progressive motility of 50 % and greater than 30 % of cells with a normal morphology.

Table 1.1. Normozoospermic semen characteristics as defined by the World Health Organisation (WHO 1992).

Standard tests	
Volume	2.0 ml
pH	7.2 - 8.0
Sperm concentration	$\geq 20 \times 10^6$ sperm/ml
Total sperm count	$\geq 40 \times 10^6$ sperm/ejaculate
Motility	≥ 50 % with forward progression within 60 minutes of ejaculation
Morphology	≥ 30 % with normal morphology
Vitality	≥ 75 % live, i.e., excluding dye
White blood cells	$< 1 \times 10^6$/ml
Optional tests	
α-Glucosidase (neutral)	≥ 20 mU per ejaculate
Zinc (total)	≥ 2.4 μmol per ejaculate
Citric acid (total)	≥ 52 μmol per ejaculate
Acid phosphatase (total)	≥ 200 mU per ejaculate
Fructose (total)	≥ 13 μmol per ejaculate

In addition to the WHO criteria, a series of nomenclature has been introduced that enables the delineation of differing fertility groups amongst men (Eliasson et al., 1970; table 1.2)

Table 1.2. Nomenclature describing semen variables in man (source: Eliasson et al., 1970).

Category	Semen characteristics
Normozoospermia	Normal ejaculate as defined in table 1.1
Oligozoospermia	Sperm concentration < 20 x 10^6/ml
Asthenozoospermia	< 50 % sperm with forward progression
Teratozoospermia	< 30 % sperm with normal morphology
Oligoasthenoteratozoospermia	Signifies disturbance of all three categories (combinations of only two prefixes may also be used)
Azoospermia	No sperm in the ejaculate
Aspermia	No ejaculate

1.1.3 *Erection and Ejaculation*

Both the central nervous system (CNS) and autonomic nervous system (ANS) control erection and ejaculation. An erection results from dilation of the arterioles of the penis. Ejaculation is a spinal reflex involving multi-glandular secretion, movement of

semen into the urethra, and ejaculation, or propulsion of the semen out of the urethra at the point of orgasm.

1.1.4 *The Blood-Testes Barrier*

The body contains a number of specialised anatomical barriers in the body, such as the blood-brain barrier and the blood-thymus barrier. In males, there also exists the blood-testes barrier. The blood-testes barrier is located somewhere between the lumen of an interstitial capillary and the lumen of a seminiferous tubule (Neaves 1977). It was first demonstrated by Setchell and co-workers (1969) that immunogoblins and iodinated albumin, inulin, and a number of small molecules were excluded from the seminiferous tubules by the blood testes-barrier. The capillary endothelium, the capillary basal lamina, the basal lamina of the seminiferous tubule, the Sertoli cells and the lymphatic endothelium are among the anatomical structures that intervene between the two luminal spaces. The barrier inhibits the free exchange of nutrients, hormones and chemicals into the lumen of the seminiferous tubule, where the germ cells are concentrated. The anatomical relationships of the blood-testes barrier may be even less developed in the immature or young mammalian testes, thereby allowing more, potentially detrimental, foreign chemicals to permeate the seminiferous tubule (Thomas 1996).

1.2 Toxic Responses of the Reproductive System

The developing gonad is very sensitive to chemical affront. In addition, the embryo is also sensitive to environmental disturbance. A concern for hazards to the

reproductive system can be traced back to the Roman Empire (Gilfillan 1965). The increased incidence of stillbirths was most likely a consequence of the high concentration of lead in pottery and water vessels. Lead is an abortifacient and is capable of producing teratospermias.

The last century has seen an enormous explosion in the number of chemicals one may be exposed too, with approximately 600 new chemicals entering commerce each year (Thomas 1996). Corresponding with the rise of the industrial production of chemicals, there has been an increase in endocrine disorders and occupational diseases (Thomas 1996). The thalidomide episode in the 1960s demonstrated the impacts of new chemicals on the reproductive system (Fabrio 1985). The incident led to an increase in awareness of the fragility of the reproductive system and the potential dangers that may arise upon exposure to chemical toxins in the work place.

Since World War II, increasing amounts of chemicals dangerous to the endocrine system have been released into the environment, and research suggests that exposure to endocrine-disrupting chemicals leads to health difficulties in a variety of animal species (Colburn et al., 1993). For example, demasculisation and defeminisation has been observed in fish, gastropods and birds, whilst in the presence of endocrine-disrupting chemicals. The reproductive system of mammals and non-mammals may also be affected by oestrogens present as natural components in the diet, in addition to environmental oestrogens (Thomas 1996).

While concerns regarding reproductive hazards have existed for centuries, most epidemiologic and reproductive factors have concentrated on maternal factors (Olshan and Faustman, 1993). It is only recently that the influence of chemical

perturbation of the male reproductive system have been studied. It has become increasingly obvious that reproductive toxicity involves both the female and male of the species (Mattison et al., 1990).

It is difficult to assess the specific potential hazards that chemicals pose to reproduction because of the complexity of the reproductive process, the unreliability of laboratory tests due to, for example, extrapolation of animal experiments, and the poor quality of human data. Human beings do, however, face numerous abnormalities in the reproductive system. For example, it is estimated that one in five couples are involuntarily sterile, over one-third of early embryos die, and about fifteen percent of recognised pregnancies abort spontaneously. Amongst the foetuses that survive, approximately three percent have developmental defects at birth, although not always anatomic. As the child ages, another six percent develop detectable defects (Thomas 1996). The above findings indicate that the reproductive system does not function in an optimal state even under normal physiological conditions. The introduction of chemicals or drugs can therefore further impede on a number of biological processes or events.

The human gonad is capable of the biotransformation of exogenous chemicals that have traversed the blood-testes barrier (Thomas 1996). For example, the primary hepatic biotransforming enzymes, the cytochrome P450 family, that are also implicated in the activation of many chemicals into toxic metabolites, have been located in testicular microsomes (Parkinson 1996). This has important toxicological implications, as many compounds are not toxic unless they are first metabolised, or activated, into a different species.

The consequence of toxic insult on the gonads is often an interference with steroidogenesis or spermatogenesis. For example, diethylhexyl phthalate and other plasticisers, produce a depletion of testicular zinc and subsequent reduction in spermatogenesis, as well as testicular atrophy (Thomas and Thomas, 1984). Metabolites of the fungicide, vinclozolin, act as antagonists of the androgen receptor (Kelce et al., 1994). The activation of 2-methoxyethanol by alcohol and aldehyde dehydrogenases to the toxin, 2-methoxyacetic acid, results in a reduction in spermatogenesis (Mebus et al., 1989). To date, however, little is known with regards to the effects of chemicals on erection or ejaculation. It has been determined that pesticides, particularly organophosphates, affect neuroendocrine processes involved in erection and ejaculation. Many drugs also produce impotence in humans, and include ethanol, hypotensives and hormones such as estrogens (Millar 1979). Occupational exposure also contributes to human male reproductive tract toxicity. Heavy metal exposure commonly correlates with reproductive pathology. For instance, lead causes reduced fertility and increased risk of offspring miscarriages and stillbirths (Thomas and Brogan, 1983). Lead also suppresses the hypothalamic-pituitary-testicular axis, thus interfering with the regulation of steroid levels (Klein et al.,1994).

It becomes apparent that a vast array of chemicals adversely affect the male reproductive system and that the sites and mechanisms of action differ greatly. This produces great difficulty in assessing the toxicity of a compound on reproductive function. Several tests are currently in use that allow the functional assessment of the male reproductive system. The majority of the tests are unacceptable for use in

humans due to the invasive nature of the assays. Table 1.3 shows a range of tests used to assess male reproductive health in animals and/or man.

Table 1.3. Some tests used to assess male reproductive toxicology in humans and/or animals (Source: Thomas 1996).

Reproductive Parameter	Test
Fertility	Number of embryos per pregnant female
	Sperm per ovum
	Ratio exposed : pregnant females
Testes	Weight, shape or size
	Histology
	Spermatid reserves
Epididymis	Weight and histology
	Number, motility and morphology of sperm at distal end
Semen	Volume
	Sperm concentration/number, motility and morphology
Sperm Motility	Multiple exposure photography
	Videomicrography
	Computer Aided Sperm Assessment (CASA)
	Cervical mucus penetration test
Endocrine	Luteinizing hormone (LH)
	Follicle stimulating hormone (FSH)
	Testosterone

The use of animals and subsequent extrapolation of data to humans represents an uncertainty due to differences in the handling, such as absorption and metabolism, and response to a chemical. Human studies are restricted depending on the toxicity of the compound in question; therefore, epidemiology studies are frequently sought to determine whether a relationship exists or not between exposure to a toxin and an adverse effect. Programs that monitor people at risk, for example industrial workers, also allow for the establishment of safer working conditions.

1.3 Important Consequences of Damage to the Reproductive System

1.3.1 *DNA Damage*

DNA attack, for example by free radicals, results in many chemical changes to DNA itself (Halliwell and Gutteridge, 1999). The production of strand breaks can arise following the exposure of DNA to reactive oxygen species (ROS). For instance, exposing DNA to ionising radiation, cigarette smoke and H_2O_2 produces strand breaks in many cell types (Halliwell and Gutteridge, 1999). In addition, ROS can produce oxidative base damage to DNA. Many methods have been detected for the measurement of several of these products, including 8-hydroxy-2′-deoxyguanosine and thymine glycol, that enable the measurement of basal levels of oxidative DNA damage and also induced production of base damage after exposure to a toxin.

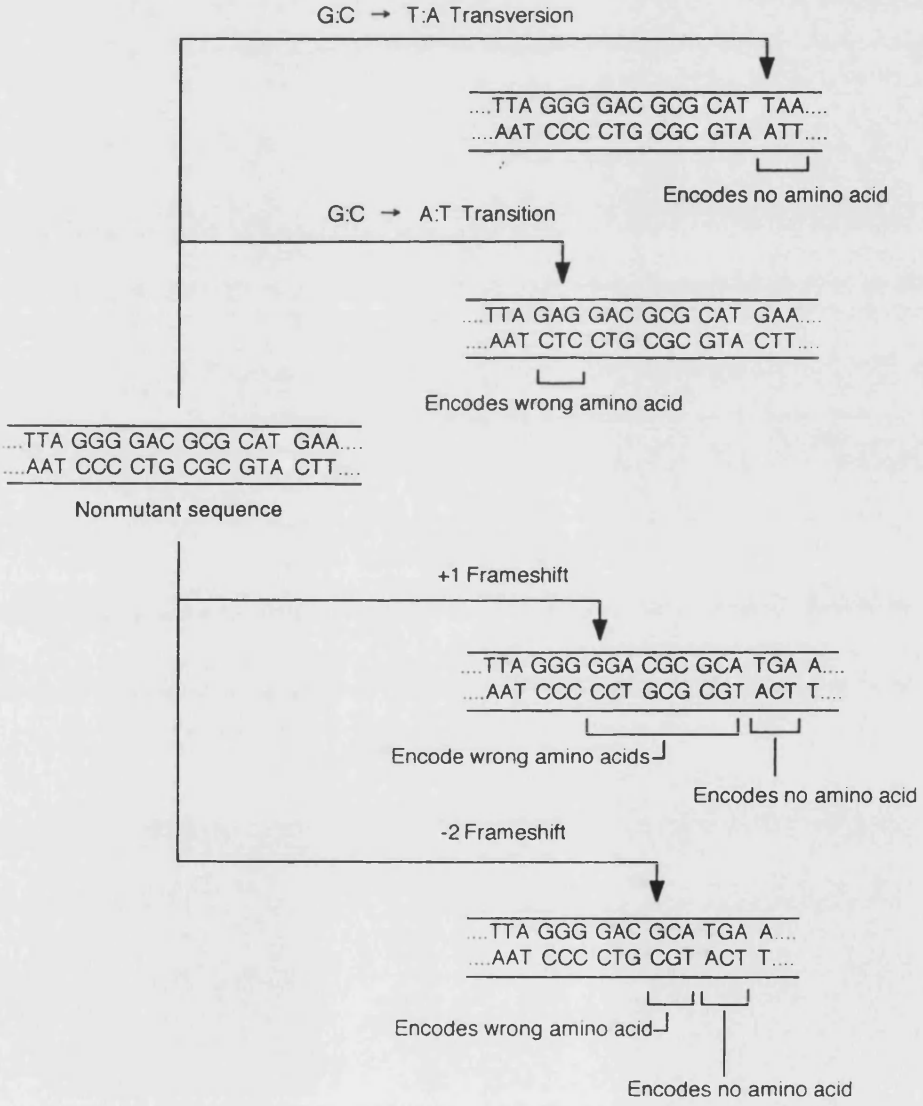
1.3.2 *DNA Damage and the Generation of Genetic Damage*

Genetic damage usually results from either gene mutations, chromosome aberrations, or changes in chromosome number.

Damage to DNA can result in mutations, either due to direct exposure to a toxin or during attempts by a cell to repair or replicate the damaged DNA (Halliwell and Gutteridge, 1999). Mutations to genes are changes in the DNA sequence of a gene; the principal forms being base-pair substitutions and frameshift mutations (Hoffmann 1996), as depicted in fig. 1.4. In a base-pair substitution, one base pair in DNA is replaced by another. The substitutions can be either transitions, whereby, for example, a purine is replaced by another, or a transversion, whereby a purine is replaced by a pyrimidine for instance. The transition or transversion may result in a missense in protein synthesis, where a coding change causes an amino acid to be replaced by another. Alternatively, a nonsense mutation may result, where the gene product is incomplete and non-functional due to the premature termination of protein synthesis. Overall, a mutation can inactivate the gene, can only have a slight effect on the gene or have no effect at all.

Frameshift mutations occur when the reading frame of the genetic code is altered during the translation of RNA into protein. Most frequently, frameshifts involve the loss or gain of one or two base pairs in a gene. The gene product involving a frameshift mutation is invariably grossly altered as every triplet in the messenger RNA is changed after the point of the mutation. In addition, the gene product is also likely to be incomplete because the new reading frame is likely to include a nonsense codon, namely UAA, UAG or UGA, which specifies no amino acid at all. Frameshift mutations generally result in non-functional gene products. Phenotypically, the consequences of a frameshift mutation will depend on how the organism is affected by the loss of that specific genes product.

Fig. 1.4. Base-pair substitutions and frameshift mutations in DNA (source: Hoffmann 1996). Arrows indicate the site of DNA mutagenesis. In this instance, the base-pair substitutions are a transversion (G:C → T:A), resulting in a nonsense mutation, and a transition (G:C → A:T), that results in a missense mutation. The frameshift mutations shown are examples in which one base pair (G:C) has been added (+1 frameshift) or two base-pairs (G:C and C:G) have been deleted (-2 frameshift). The frameshift mutations alter the reading frame of the genetic code for all codons after the point of the mutation.



Changes in chromosome structure that involve large alterations in the genetic material are termed chromosomal aberrations. Damage may include chromosome breakage or chromosome breakage and subsequent rearrangement. Most aberrations are unstable and result in the death of the cell, although some persist and are transmitted as cell division occurs.

Aneuploidy and polyploidy represent the cellular consequences of changes in chromosome number. Aneuploidy involves a deviation from the normal diploid number, whereas polyploidy involves a complete loss or gain of a set of chromosomes. For example, a human somatic cell possessing 47 chromosomes would be defined as aneuploid. A cell possessing 69 chromosomes would be said to be polyploid, in this instant triploid, or three times the haploid number.

The adverse consequences of gene mutations in germ cells is evident from the number of genetically inherited diseases. Around 1.3 % of neonates are born with autosomal dominant (~1 %), autosomal recessive (~0.25 %) or sex-linked (~0.05 %) genetic diseases (Sankaranarayanan 1993). As well as inherited disease, gene mutations can produce congenital abnormalities and increased frequencies of foetal death during development (Sankaranarayanan 1993). Chromosomal abnormalities can also result in foetal death or serious disease states.

1.3.3 *Lipid Peroxidation*

Lipid peroxidation occurs when polyunsaturated lipids become damaged due to oxidative stress (Halliwell and Gutteridge, 1999). The major constituents of biological membranes are protein and lipid, with the lipid component commonly

containing polyunsaturated side-chains (Halliwell and Gutteridge, 1999). The oxygen-dependent process of lipid peroxidation results in lipid rancidity; organisms, therefore, must be adequately protected against such phenomena.

1.3.4 *The Production of Lipid Peroxidation: Initiation and Propagation*

The sequence leading to lipid peroxidation in biological systems is a multi-step process, ultimately resulting in the generation of detrimental lipid peroxides (see Halliwell and Gutteridge, 1999).

Initiation of lipid peroxidation occurs with the extraction of a hydrogen atom from a methylene group in a polyunsaturated fatty acid by a sufficiently active species, such as a hydroxyl radical:

$$-CH_2- + OH^{\bullet} \rightarrow -^{\bullet}CH- + H_20$$

the removal of a hydrogen from a methyl group leaves behind an unpaired electron on the carbon. The presence of a double bond in the lipid reduces the C–H bonds on the carbon atom adjacent to the double bond, thus aiding removal of the hydrogen atom. The formed carbon radical usually becomes stabilised by a molecular rearrangement, producing a conjugated diene. The most common fate of the carbon radical in an aerobic system is to react with oxygen to produce a peroxyl radical, $ROO^{\bullet}$:

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$$

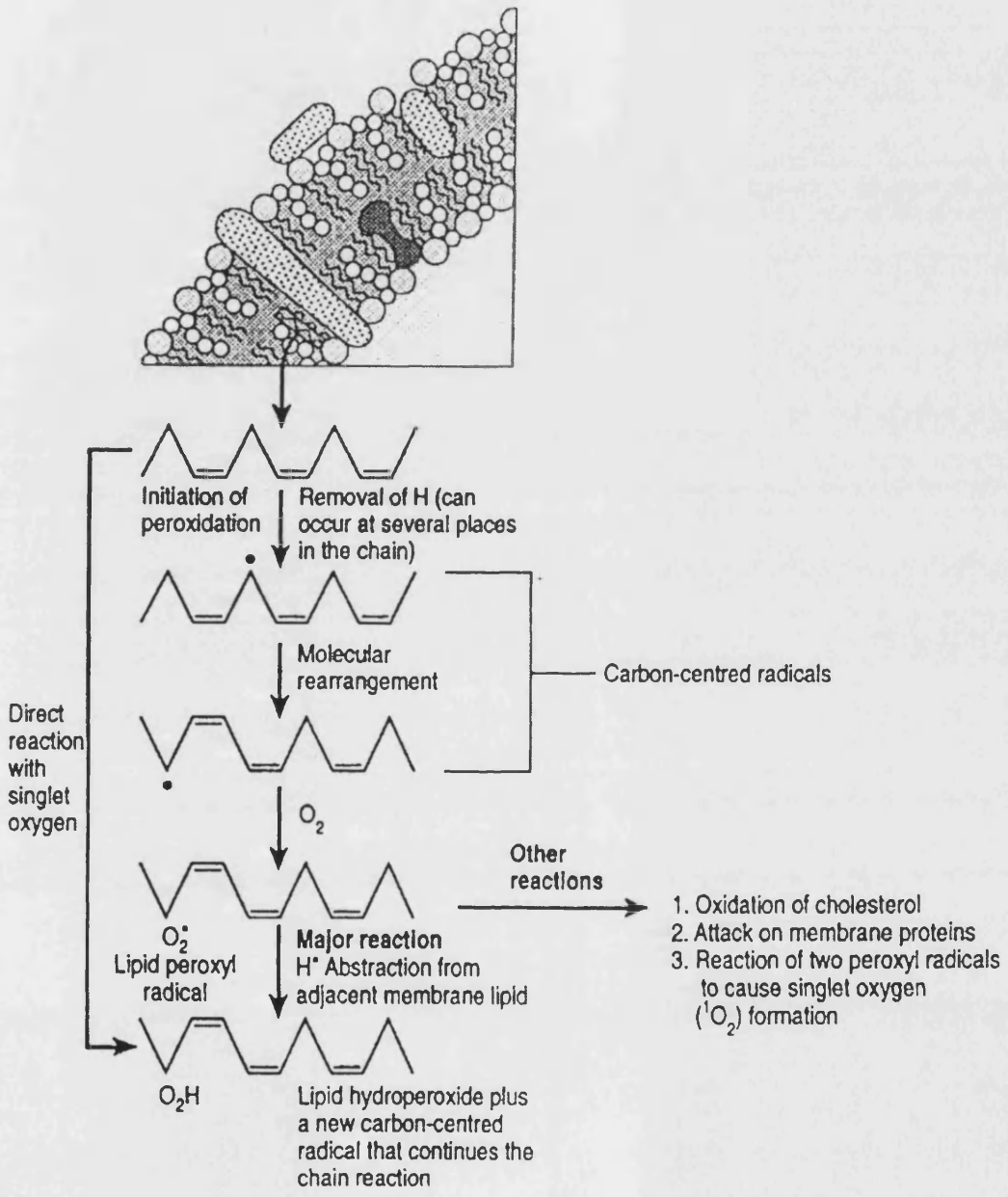
The peroxyl radical is then able to extract a hydrogen atom from another lipid molecule, resulting in the *propagation* of lipid peroxidation:

$$ROO^{\bullet} + CH \rightarrow -ROOH + C^{\bullet}$$

The carbon radical so formed can now react further with oxygen to form another peroxyl radical, and so the chain reaction continues.

The peroxyl radicals formed during the propagation steps combines with the hydrogen atom it abstracts to produce a lipid peroxide. In addition to the formation of deleterious lipid peroxides, two end-products of lipid peroxidation, malondialdehyde and 4-hydroxy-2-nonenal, are known to produce mutagenic DNA adducts (Halliwell and Gutteridge, 1999). An example of the generation of lipid peroxidation in a fatty acid with three double bonds is provided in fig. 1.5.

Fig. 1.5. Proposed mechanism for the production of lipid peroxides from a fatty acid possessing three double bonds (source: Halliwell and Gutteridge, 1999).



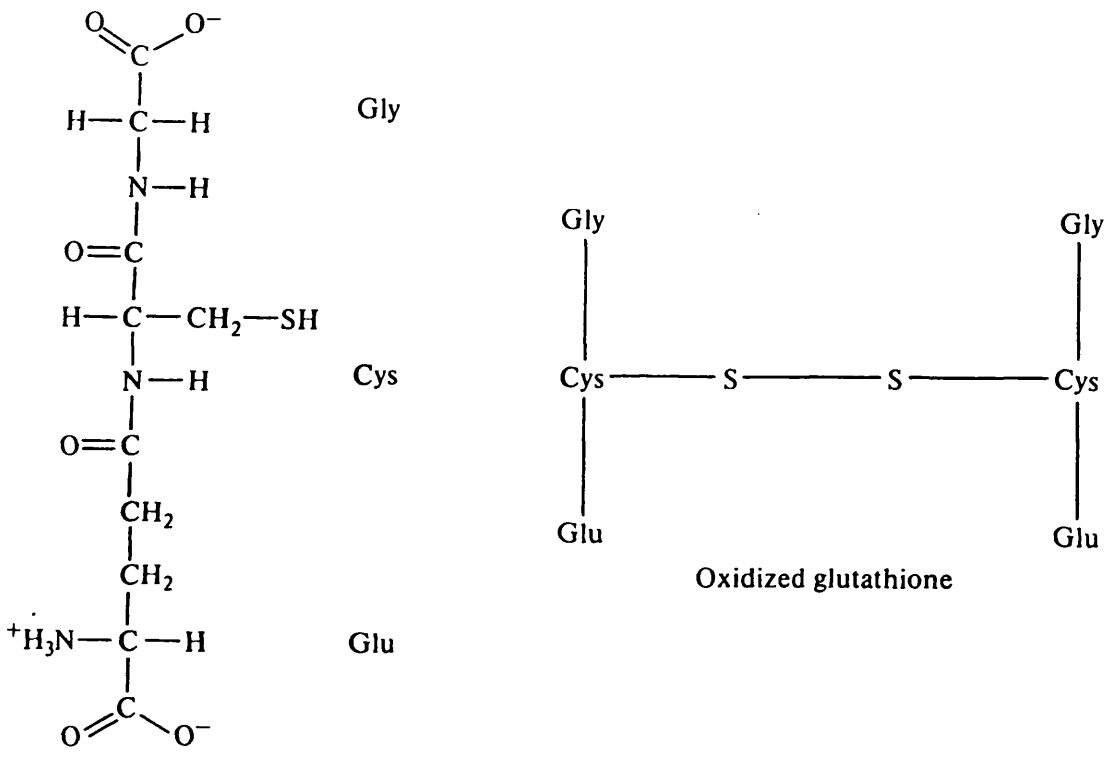
1.4 Antioxidants

Antioxidants are well established in the biological sciences as playing a crucial role in the reduction of oxidative attack. An antioxidant can be defined as 'any substance that, when present at low concentrations compared to that of an oxidisable substrate, significantly delays or inhibits oxidation of that particular substrate' (Halliwell and Gutteridge, 1999). Antioxidants are of importance because oxidation of a substrate and the production of electrophiles and radical species, toxic intermediates, can lead to binding by the reactive compound to critical nucleophiles, such as nucleic acids and proteins, and produce genetic and other cellular damage. Biological actions of antioxidants include scavenging initiating radical species and also intermediate radical species; the latter being so-called chain-breaking antioxidants because of their capability to prevent further hydrogen abstraction and continued radical formation. Many enzymes also act alone and in combination to detoxify radicals. The modes of action of several important antioxidants will now be discussed.

1.4.1 *Glutathione*

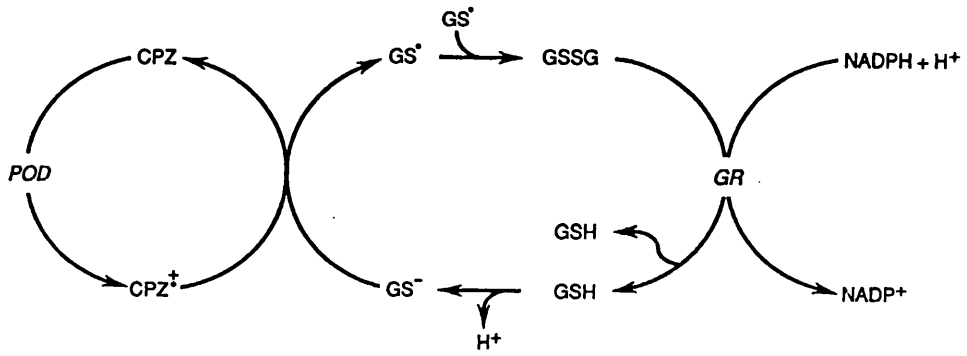
Glutathione (GSH) is a thiol containing tripeptide, composed of a cysteine (Cys), glutamic acid (Glu) and glycine (Gly) residue, as depicted in fig. 1.6.

Fig. 1.6. Structure of glutathione in both its reduced (GSH) and oxidised (GSSG) form (source: Halliwell and Gutteridge, 1999).



GSH detoxifies radical species by donating hydrogen atoms and reducing compounds; and by forming conjugates, catalysed by the family of enzymes, glutathione *S*-transferases, although non-enzymic conjugation does also occur, albeit at a much slower rate (Parkinson 1996). An example of a typical detoxification reaction by GSH is depicted in fig. 1.7.

Fig. 1.7. A typical detoxification reaction performed by glutathione (GSH) (source: Gregus and Klaassen, 1996). In this instance, peroxidase (POD) metabolism of chlorpromazine (CPZ) produces a free radical species, $CPZ^{+\bullet}$. GSH detoxifies the free radical by the donation of an electron. The glutathione thiyl radical ($GS^{\bullet}$) combines with another $GS^{\bullet}$ spontaneously, forming glutathione disulphide (GSSG). GSH is then regenerated from GSSG by the actions of glutathione reductase (GRD).



GSH reduces the reactive species and becomes its oxidised form, GSSG, whereby two GSH molecules are joined to produce a disulphide bridge (fig. 1.6). The ratio of GSH : GSSG is kept high in vivo; this is accomplished via the enzyme glutathione reductase (GRD), which regenerates GSH by the reaction below:

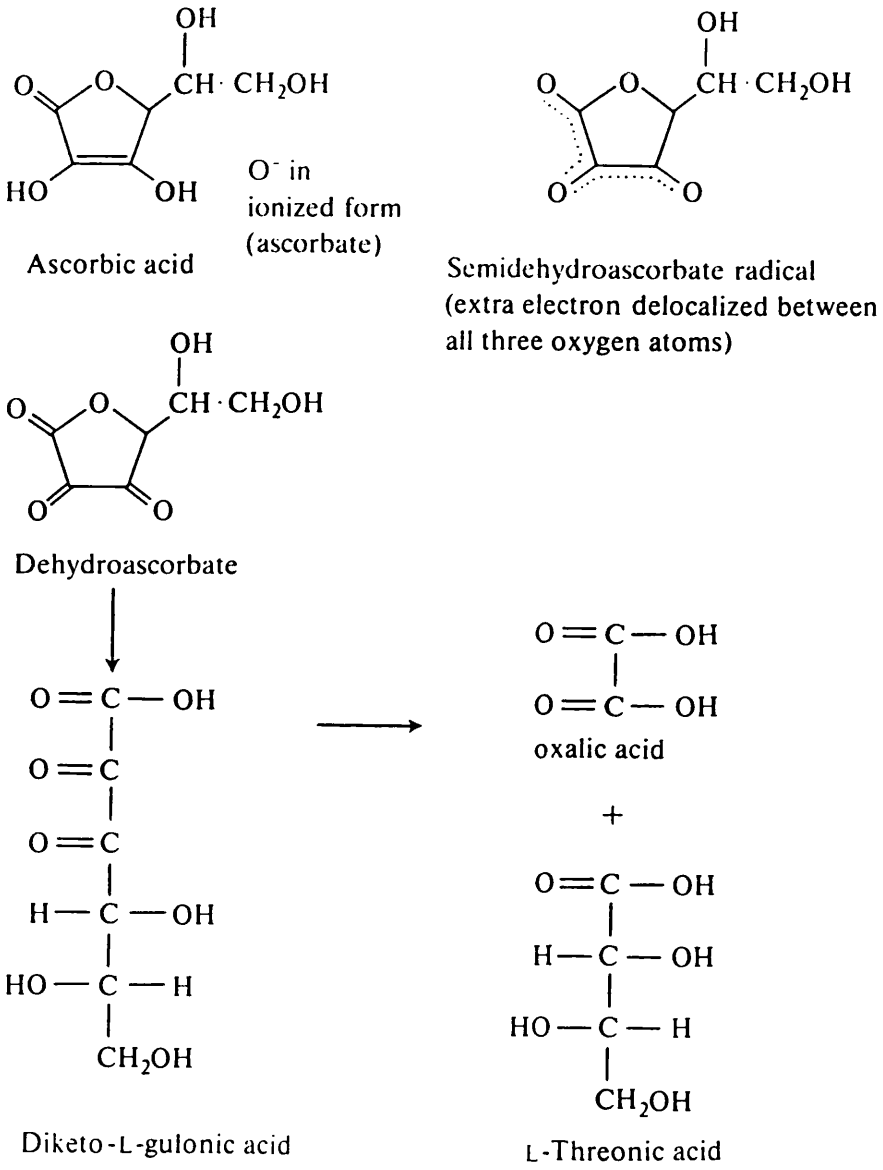
$$GSSG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+$$

GSH is present in animals and plants, as well as certain bacteria, often at millimolar concentrations in certain tissues such as the liver. In human seminal plasma, GSH is present at a concentration of 0.5 μM (Li 1975; Ochsendorf et al., 1998). The compound is also present in very small amounts in human spermatozoa, at levels of around 3 nmol/10^8 spermatozoa (Li 1975; Ochsendorf et al., 1998).

1.4.2 *Ascorbic acid*

Ascorbic acid, or vitamin C, is a water soluble solid, that many plants and animals can synthesise in vivo. Man, however, is not able to produce the compound and is, therefore, reliant on ascorbic acid to be present in the diet. Fig. 1.8 shows the reduced (ascorbate) and oxidised (dehydro-L-ascorbate) forms of ascorbic acid. Ascorbate can act as a reducing agent, and it is this property which gives ascorbic acid its antioxidant properties (Halliwell and Gutteridge, 1999). For example, ascorbate is capable of donating a hydrogen atom to peroxyl radicals, thus forming non-radical species (Gregus and Klaassen, 1996).

Fig. 1.8. Structures of ascorbic acid in its reduced and oxidised forms, as well as the products of ascorbate degradation, diketo-L-gulonic acid, oxalic acid and L-threonic acid (source: Halliwell and Gutteridge, 1999).



1.4.3 *Enzymic Oxidant Protection*

Numerous enzymes play key roles in the antioxidant defences of a species, often working together to ultimately detoxify a radical (see Halliwell and Gutteridge, 1999). For example, superoxide dismutase (SOD) is an enzyme found in virtually all eukaryotic cells and in every instance, catalyses the dismutation of superoxide, a toxic reactive intermediate, as in the reaction below:

$$O_2^{\cdot} + \ + 2H^+ \rightarrow H_2O_2 + O_2$$

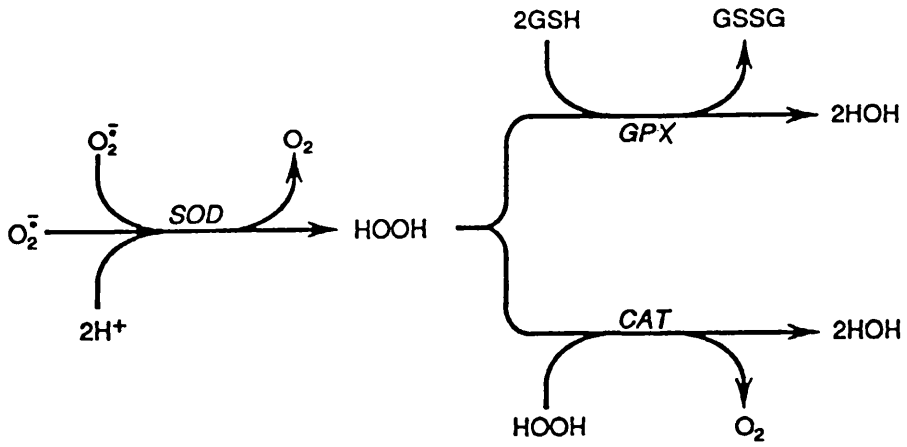
SOD is present in man in several forms: copper-zinc-SOD (Cu,Zn-SOD) is found in mainly cellular cytosol; manganese-SOD (Mn-SOD) is located primarily in the mitochondria; and a third form is also found extra-cellularly, EC-SOD. Catalase (CAT) is another enzyme found in many organisms and is present in most major organs in man. The enzyme catalyses the breakdown of hydrogen peroxide, a toxic oxidant, into water and oxygen.

Glutathione peroxidase (GPX) utilises GSH as its substrate, in order to detoxify hydrogen peroxide, according to the below reaction:

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$$

Together, these enzymes often act in concert to detoxify products of oxidative stress, as outlined in fig. 1.9.

Fig. 1.9. Detoxification of the superoxide anion radical ($O_2^{\bullet -}$) by superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) (source: Gregus and Klaassen, 1996).



In combination, each component of an antioxidant defence system produces an effective mechanism to protect that organism. However, in certain circumstances detoxification fails and damage ensues. Detoxification may fail, for example, when depletion of antioxidants such as glutathione or vitamin E occurs. Similarly, if excess toxins accumulate in the organism, protective enzymes may become saturated and their sources of cofactors, necessary for normal functioning, exhausted.

1.4.4 *Antioxidants in Seminal Fluid*

Seminal fluid contains several antioxidants commonly found in blood plasma, albeit in many cases, at differing concentrations. Due to the importance of protecting sperm from radical attack, and thus maintaining fertility status and genetic integrity, many studies have investigated the antioxidant capacity of sperm and seminal plasma.

Seminal plasma contains high levels of ascorbic acid (400 μmol/L) compared to blood plasma (40 μmol/L) (Fraga et al., 1991; Thiele et al., 1995; Lewis et al., 1997), which would suggest that ascorbate has an important physiological role in the male reproductive tract. Indeed, ascorbate has been shown to be negatively correlated with sperm oxidative DNA damage (Fraga et al., 1991), seminal plasma and spermatozoal ROS production (Thiele et al., 1995; Lewis et al., 1997), and abnormal sperm morphology (Thiele et al., 1995).

Another important antioxidant that is a component of the diet in man is Vitamin E, comprising a group of four tocopherols. Vitamin E is a lipophilic compound, particularly efficient at protecting against lipid peroxidation through its reactions with alkoxy and lipid peroxy radicals, donating a hydrogen to them (Halliwell and Gutteridge, 1999). The vitamin E radical formed after such a reaction is not reactive enough to abstract a hydrogen from lipids, thus terminating the chain reaction of peroxidation. Seminal plasma is a rich source of vitamin E, which is not surprising in light of the particularly deleterious actions of lipid peroxidation on sperm, namely its associations with reduced fertility (Suleiman et al., 1996).

Uric acid is another antioxidant present in large amounts in seminal plasma, at concentrations of around 200 μM, similar levels to those found in blood plasma

(Halliwell and Gutteridge, 1986; Lewis et al., 1997). Urate is a powerful scavenger of oxidants, again reflecting the importance of protecting sperm from the deleterious actions of radicals. Thiol containing compounds, including GSH, are also found in seminal plasma in large amounts, mainly associated with protein (Lewis et al., 1997). The protective enzymes CAT, SOD, GPX and GRD have also been detected in seminal plasma (Kobayashi et al., 1991; Sanocka et al., 1996; Alkan et al., 1997; Yeung et al., 1998). Additionally, CAT and SOD have been found at low levels in spermatozoa (Sanocka et al., 1997).

In addition to the high levels of antioxidants present in seminal plasma, spermatozoa themselves possess thiol groups, although substantially less than those found in seminal plasma, as well as less substantial amounts of ascorbic acid, vitamin E, uric acid and GSH (Li 1975; Lewis et al., 1997; Ochsendorf et al., 1998).

In conclusion, spermatozoa do not possess significant amounts of antioxidants. This produces a dependence of sperm on their extracellular environment for protection from oxidative stress; a requirement reflected in the abundance of antioxidants found in seminal plasma.

1.5 Cigarette Smoking and Reproduction

Cigarette smoking has escalated despite being one of the major risk factors to the general health of the public. By 1988, it was estimated that one billion people consumed an annual total of 5.2 trillion cigarettes world-wide (Connolly 1992). The consumption of cigarettes by men varies according to the region of the world, but is somewhere between 30 and 80 % (Council on Scientific Affairs 1990).

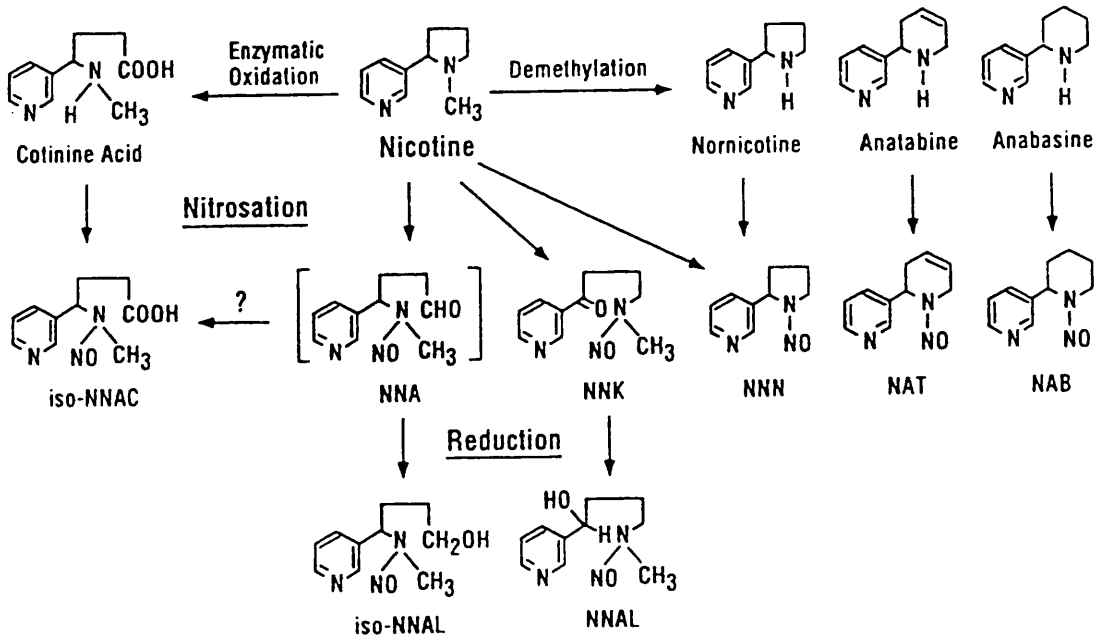
1.5.1 *The Composition of Cigarette Smoke*

Cigarette smoke and its condensates are comprised of many hundreds of potentially hazardous chemicals, including numerous free radical species (Halliwell and Gutteridge, 1999). Cigarette smoke itself contains over 4000 components, of which more than 50 are animal mutagens and carcinogens (Hoffmann and Hecht, 1989; Brunnemann and Hoffmann, 1992). The carcinogens can be divided into their respective classes; the major animal carcinogens being the nitrosamines, including tobacco-specific nitrosamines (TSNA) and volatile nitrosamines, polycyclic aromatic hydrocarbons (PAH) and heterocyclic and aromatic amines. These compounds are implicated in the genesis of several cancers in man, most commonly lung cancer, followed by cancer in the colon and rectum, but also cancers of the oral cavity, pancreas, kidney and urinary bladder, amongst others (Pitot and Dragan, 1996).

Tobacco, obtained from the plant *Nicotinia*, contains around 40 individual alkaloids, the majority of which are 3-pyridyl alkaloids. Nicotine is the principal alkaloid in tobacco, comprising as much as 95 % of the total alkaloid content, and is a highly addictive compound in man.

The alkaloids present in tobacco are either secondary or tertiary amines, which are capable of undergoing nitrosation. Fig. 1.10 shows a proposed mechanism for the formation of several TSNA through the enzymic metabolism of nicotine and other alkaloids present in tobacco smoke in vivo.

Fig. 1.10. Proposed mechanism for the production of tobacco-specific nitrosamines (TSNA) in vivo (source: Brunnemann et al., 1996).



The rate of nitrosation is variable (Brunnemann et al., 1996). Generally, nitrosation is slow in vivo. However, the drying, curing and ageing of tobacco prior to human consumption can lead to the reduction of nitrate to nitrite and subsequent formation of TSNA following nitrosation. Ultimately, levels of nitrosamines in mainstream cigarette smoke vary according to the nicotine content and type of filter present on the cigarette; mainstream smoke being that which is inhaled during human consumption. High tar and nicotine cigarettes, containing around 12 mg tar and 1.9 mg nicotine, yielded 4-(methylnitrosamino-)-1-(3-pyridyl)-1-butanone (NNK) levels of 17 to 156 ng/cigarette and N-nitrosonornicotine (NNN) levels of 49 to 287 ng/cigarette. Lower tar and nicotine containing cigarettes contain accordingly, significantly lower levels of nitrosamines.

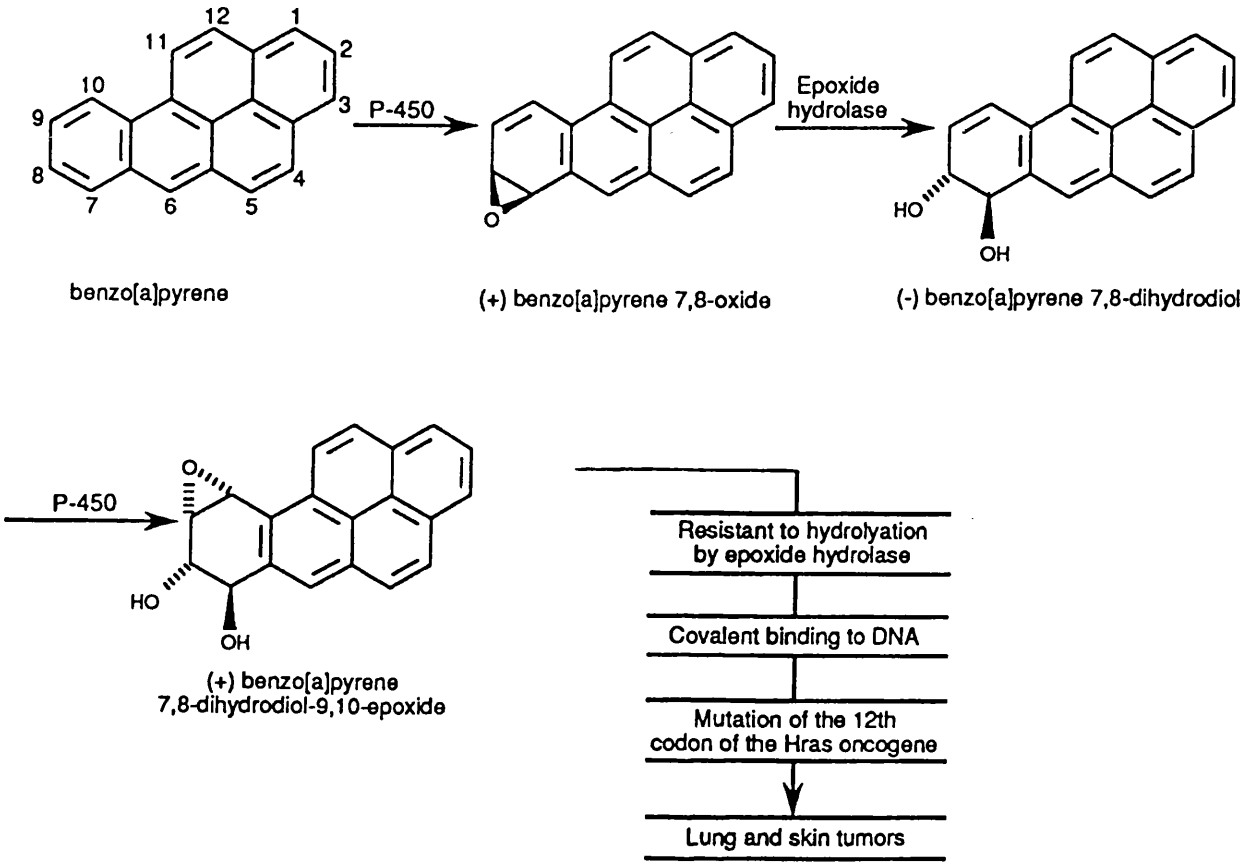
PAH, formed by the combustion of carbonaceous materials, and aromatic and heterocyclic amines are present in both mainstream and sidestream tobacco smoke (Pitot and Dragan, 1996). These classes of compounds also represent several known or suspected mutagens and carcinogens in man (Pitot and Dragan, 1996).

Metabolism of the above compounds in vivo may result in the formation of reactive intermediates, that, potentially, are capable of damaging DNA and producing mutagenesis. This process is termed metabolic activation. Metabolic activation is invariably by one or more of the cytochrome P450 isofamily, prevalent throughout many tissues in the human body (Wrighton et al., 1996), including the testes (Parkinson 1996).

The TSNA are metabolically activated by hydroxylation, leading to the formation of several unstable and reactive intermediates (Parkinson 1996). Many intermediates

are so unstable they spontaneously form other compounds which are capable of reacting with DNA. Upon reacting with DNA, methylation of a nucleotide often ensues, that may result in mutagenesis. Aromatic amines are activated by transformation to a reactive N-hydroxy intermediate, capable of producing other, more reactive species, that can produce DNA damage (Parkinson 1996). The toxicity of PAH is commonly through metabolism into toxic epoxides, which are capable of forming DNA adducts (Parkinson 1996). An example of the metabolic conversion of a polycyclic aromatic hydrocarbon, in this instant benzo[a]pyrene, by cytochrome P450 and epoxide hydrolase to a tumourigenic diol-epoxide compound, is depicted in fig. 1.11.

Fig. 1.11. The metabolic conversion of benzo[a]pyrene to its mutagenic and carcinogenic diol-epoxide (source: Parkinson 1996).



Metals are also present in cigarette smoke that are also of toxicological interest, including cadmium, iron, arsenic, nickel, mercury, chromium, *cis*-platinum, beryllium and lead (Goyer 1996; Smith et al., 1997). Metals differ from other toxins in cigarette smoke previously discussed in that they are not created by tobacco combustion, but are present at residual levels in the soil and atmosphere where they may be absorbed by the tobacco plants. Several metals that are present in cigarette smoke have been

smoke have been associated with mutagenesis and carcinogenesis in animals and/or man, including cadmium, lead, nickel, arsenic, chromium and beryllium (Goyer 1996; Smith et al., 1997).

1.5.2 *Smoking and Male Reproductive Toxicology*

Due to the mutagenicity of cigarette smoke, numerous studies have investigated the impact of male smoking on sperm, which have been reviewed several times (Mattison 1982; Stillman et al., 1986; Little and Vainio 1994; Vine 1996). Smoking is associated with an overall reduction in semen quality, specifically a reduction in sperm count and motility, and an increase in the number of abnormal cells (Pacifici et al., 1993; Vine et al., 1994; Sofikitis et al., 1995; Vine et al., 1996). Additionally, smoking has been associated with increased levels of aneuploidy in human sperm (Wyrobek et al., 1995), lower seminal plasma anti-oxidant levels, and increased oxidative damage to sperm DNA (Fraga et al., 1996; Shen et al., 1997).

Vine (1996) conducted a study analysing data from 27 separate reports that analysed male smoking and fertility. It was concluded that smokers showed a 13 % reduction in both sperm concentration and sperm motility compared to non-smokers, and a mean increase of 3 % in the number of abnormal sperm. Results of studies investigating semen quality and cigarette consumption are, however, not always in unison. For example, Vine et al. (1996) found a significant inverse correlation between sperm concentration and the number of cigarettes smoked per day and serum, semen and urine cotinine levels. Cotinine being a major metabolite of nicotine produce in vivo. However, Pacifici et al. (1993) found no such correlation

between seminal nicotine and cotinine levels and cell concentration. Overall, it can be concluded that the majority of studies have found a negative association between sperm count, motility and morphology and cigarette smoking. It also appears that semen quality amongst smokers and non-smokers varies between healthy men and those with low fertility, with healthy men being more susceptible to the adverse effects of cigarette smoking (Vine 1996). The reason for this discrepancy is not known.

Several cancers of the reproductive system have been associated with male smoking by epidemiological studies. A direct action on the male is apparent, as prostate and testicular tumours are all more aggressive, if not more prevalent in smokers (Daling et al. 1992). In addition, an adverse effect on the partner of a male smoker has been reported; namely an increased risk of cervical cancer (Bosch et al., 1996).

There is also epidemiological evidence that pre-conceptual paternal smoking is associated with adverse effects on his offspring, as distinct from effects due to maternal and offspring passive smoking. Zhang et al. (1992) reported an overall elevated risk of birth defects in paternal smokers and concluded in their survey that paternal smoking may result in multiple rather than individual birth defects. Savitz et al. (1991) found certain abnormalities were positively associated with male smoking whilst others were negatively associated. Recent studies have also implicated pre-conceptual male smoking with enhanced risk of certain childhood cancers (Sorahan et al., 1995; 1997a; 1997b).

The toxic effects of metals that are present in cigarette smoke, industry and the environment on the male reproductive tract are also of concern (Thomas 1996). Lead

exposure can result in infertility, sterility and increased levels of sperm morphological defects. Cadmium, iron, mercury, silver, cobalt, *cis*-platinum and molybdenum can adversely affect accessory gland function and spermatogenesis. In addition, deficiencies in the levels of essential metals, such as zinc and selenium, is associated with a reduction in sperm count and motility.

1.6 Aims and Scope of the Project

It has been reported that male fertility has decreased by thirty percent over the last fifty years (Carlsen et al., 1992). However, the causative factors that could account for such a decrease are not yet well established. In addition, the quality of semen is increasingly under threat and factors that increase the incidence of sperm mutations and production of germline abnormalities which can result in birth defects and genetic disease in offspring, are an important area of study in order to protect human populations.

The aims of the study were to compare semen from smokers and non-smokers, in order to determine whether cellular differences exist between the two groups. In particular, the extent of DNA damage in the cells will be investigated, since that may predispose offspring to an increased risk of genetic anomalies. The production of mutations in sperm, according to the generation of aneuploidy, will also be ascertained. Additionally, potential associations between smoking and reduced fertility will be investigated.

Antioxidants in both seminal plasma and the spermatozoa themselves play an integral role in the protection of sperm and maintenance of male fertility. Cigarette smoking is known to produce oxidative stress and reduce the abundance of antioxidants in blood plasma (Halliwell and Gutteridge, 1999). Antioxidant levels will, therefore, be determined in the seminal fluid of smokers and non-smokers so as to determine whether smoking produces lower antioxidant capacity and increased levels of oxidative stress in semen, that may correlate with cellular damage and reduced fertility.

The epididymis acts as a site for the storage and maturation of sperm. The antioxidant activity of the epididymal fluid will be determined, as well as the ability of the epididymis to support spermatozoal subsistence, by comparing the ejaculate of normozoospermic and vasectomized individuals.

Finally, an analytical assessment of seminal fluid will be performed in order to determine whether compounds are present that are capable of being beneficial to, or adversely affect, the male reproductive system and which could result in reduced fertility and/or offspring pathology.

2. Sperm chromatin damage associated with male smoking

2.1 Introduction

Cigarette smoking is associated with several adverse effects on the male reproductive system, including a reduction in sperm count and motility and an increase in the number of abnormal cells (Pacifici et al., 1993; Sofikitis et al., 1995; Vine et al., 1994; Vine et al., 1996). Smoking has also been associated with oxidative damage to sperm DNA (Fraga et al., 1996; Shen et al., 1997), and higher levels of sperm aneuploidy (Wyrobek et al., 1995).

Certain epidemiological studies have also implied that paternal pre-conceptual smoking may be associated with adverse effects on offspring. Sorahan et al. (1995; 1997a; 1997b) found paternal pre-conceptual daily cigarette consumption produced an overall increase in all childhood cancers. Zhang et al. (1992) also reported an overall elevated risk of birth defects in offspring of paternal smokers. The measurement of DNA damage in spermatozoa is therefore critical in order to evaluate the potentially adverse effects of exposure to toxins, such as those found in cigarette smoke.

Several methods are available that enable the measurement of spermatozoal DNA damage.

2.1.1 *Alkaline Single Cell Gel Electrophoresis Assay*

The alkaline single cell gel electrophoresis, or 'comet', assay, is a sensitive visual technique that allows the measurement of DNA single and double strand breaks

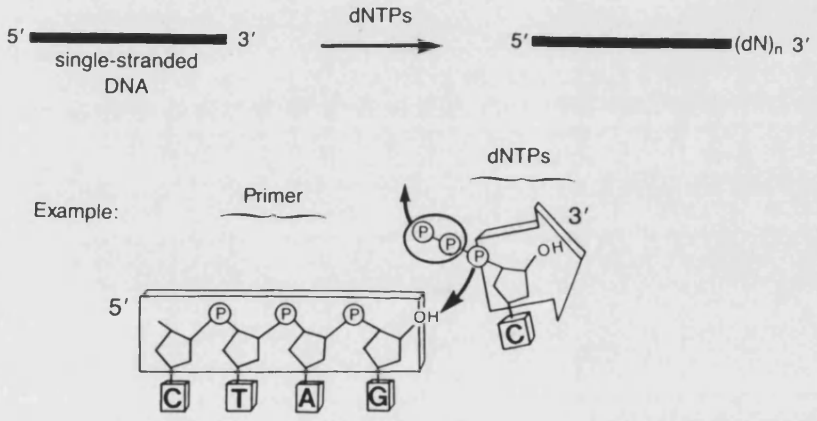
(Singh et al., 1989; McKelvey-Martin et al., 1993), and it has been applied to the determination of DNA damage in human sperm (McKelvey-Martin et al., 1993; Hughes et al., 1997). Cells are lysed, embedded in agarose on a microscope slide and electrophoresed under alkali conditions to promote unwinding of DNA. Cells are then stained with a nucleic acid dye, such as ethidium bromide, and viewed under fluorescence microscopy. Cells that contain DNA strand breaks cause the release of DNA supercoiling and allow the migration of DNA from cells. This produces the appearance of a 'comet' in such cells; the longer the comet tail, the greater number of strand breaks present in the cell. Fluorescent microscopy and image analysis software enable the quantification of the amount of damage present in a cell. The assay can be used to quantify DNA damage and repair of DNA, and it can determine whether all the cells showed the same degree of damage in a sample or whether a heterogeneous response occurs.

2.1.2 *Terminal Deoxynucleotidyl Transferase Assay*

The terminal deoxynucleotidyl transferase assay (TdTA), sometimes referred to as the TUNEL (TdT-mediated X-dUTP nick end-labelling) assay, allows the measurement of DNA strand breaks, through the addition of the biotinylated nucleotide dUTP to 3'-OH ends of DNA, sites of DNA breakage, using the enzyme terminal deoxynucleotidyl transferase (TdT; Gorczyca et al., 1993). Fig. 2.1 outlines the reaction occurring using the TdTA and a broken strand of DNA. The biotinylated nucleotide is labelled with fluoresceinated avidin (FITC-avidin) and its emissions are

then determined using either flow cytometry or fluorescence microscopy, in order to ascertain the proportion of cells containing strand breaks.

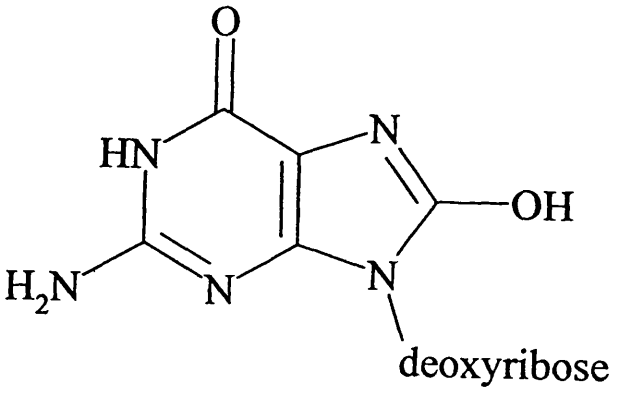
Fig 2.1. Diagram depicting the terminal deoxynucleotidyl transferase assay (TdTA). DNA strand breaks are measured through addition of the biotinylated nucleotide dUTP to 3′-OH ends of DNA, sites of DNA breakage, using the enzyme terminal deoxynucleotidyl transferase (TdT). The biotinylated nucleotide is labelled with fluoresceinated avidin (FITC-avidin) and its emissions can then be determined using either flow cytometry or fluorescence microscopy, in order to ascertain the number of cells containing strand breaks.



2.1.3 *Determination of 8-hydroxy-2'-deoxyguanosine*

Measurement of 8-hydroxy-2'-deoxyguanosine, or 8-OHdG (fig. 2.2), one of around twenty major oxidative DNA damage products, has been conducted in human sperm (Fraga et al., 1991; Fraga et al., 1996; Shen et al., 1997). The lesion is produced through the oxidative attack of guanine.

Fig. 2.2. Structure of 8-hydroxy-2'-deoxyguanosine, one of approximately twenty major products of oxidative DNA attack.



Levels of 8-hydroxy-2′-deoxyguanosine have been estimated at a total of 5 x 10^5 oxidative adducts per spermatozoon (Fraga et al., 1991).

2.1.4 *Sperm Chromatin Structure Assay*

The sperm chromatin structure assay (SCSA) measures the susceptibility of sperm cells to acid or heat induced denaturation in situ (Evenson and Jost, 1994; Sailer et al., 1995). During human sperm maturation, nuclear histones are replaced by arginine/cysteine-rich protamines, followed by chromatin stabilisation through intra- and inter-protamine cross-linking via disulphide bridges in the epididymis (Bedford 1979; Balhorn 1989). This process produces a highly condensed sperm nucleus that is more resistant to DNA cleavage and denaturation and in situ enzymatic degradation than a somatic cell. The SCSA measures the production of single stranded DNA (ssDNA) after acid or heat treatment and staining of cells using the metachromatic stain acridine orange (Evenson and Jost, 1994; Sailer et al., 1995).

The SCSA has been correlated with exposure to several mutagenic compounds (Evenson and Jost, 1993; Evenson et al., 1993a, 1993b). The assay also shows strong correlations with DNA strand breaks in human and other mammalian spermatozoa, determined using the TdTA (Sailer et al., 1995; Aravindan et al., 1997) and alkaline single cell gel electrophoresis assay (Aravindan et al., 1997). In addition, the assay is an effective predictor of fertility (Evenson et al., 1999). The SCSA provides results of a lower variability within an individual than any other standard semen variable and it is an objective, technically sound, biologically stable, sensitive and feasible measure of semen quality (Evenson et al., 1991).

The present study analysed the DNA of sperm obtained from smoking and non-smoking subjects, with a normozoospermic semen profile, using the SCSA and TdTA assay The purpose of this report is to document that more damage is present in the nuclear material of sperm from smokers compared to that of non-smokers, in the form of increased susceptibility to in situ DNA denaturation and higher levels of DNA strand breaks.

2.2 Methods

2.2.1 *Semen Samples*

Semen samples were obtained from patients being screened for fertility at the Royal United Hospital, Bath, UK. All samples were collected by masturbation after 3 days of abstinence. Following liquefaction at room temperature for 1 h, semen analysis was performed by staff of the Royal United Hospital (only those classified as normozoospermic as defined by the World Health Organisation (WHO 1992) were included in the study). Normozoospermic samples were defined as possessing $\geq 20 \times 10^6$ spermatozoa/ml, forward progressive motility $\geq 50\%$, normal morphology $\geq 30\%$ and $< 1 \times 10^6$ white blood cells/ml. Samples were then stored at -20°C prior to use. The smoking status of each donor was recorded and the samples used in the study characterised as either non-smokers or smokers of more than 10 cigarettes per day. This study had the approval of the Bath District Ethics Committee.

2.2.2 *SCSA*

The SCSA measures the ratio of single stranded DNA (ssDNA; red fluorescence at (630 nm) to double stranded DNA (dsDNA; green fluorescence at 515-530 nm) after acid treatment and staining of cells using the metachromatic stain acridine orange (Evenson and Jost, 1994; Sailer et al., 1995). The higher the ssDNA : dsDNA ratio, the greater the degree of denaturation and the more fragile the chromatin structure. The change in gradient of the main population of cells taken from the scatter plot before and after acid treatment was used as a measure of denaturation; as dsDNA

converts to ssDNA, the slope decreases. The SCSA was performed on 10000 cells from each of 35 non-smokers and 35 smokers and analysed by flow cytometry.

Two hundred microlitre aliquots of sperm (1-2 x 10^6/ml) were admixed with 0.40 ml of 0.1 % Triton X-100, 0.15 M NaCl and 0.08 N HCl, pH 1.4. After 30 seconds, the pH in the cell suspension was raised and the cells were stained by adding 1.2 ml of acridine orange buffer containing 6 μg/ml acridine orange, 370 ml 0.1 M citric acid, 630 ml 0.2 M Na_2HPO_4, 1 mM disodium EDTA and 0.15 M NaCl, pH 6.0 (Evenson and Jost, 1994; Sailer et al., 1995). Cells were analysed by flow cytometry using a laser light excitation of 488 nm.

2.2.3 *TdTA*

Cells are permeabilised, fixed and subjected to exogenous TdT enzymes in the presence of biotinylated 16-dUTP. The nucleotide is added to the 3'-OH ends of DNA strand breaks by TdT and detected by FITC-avidin. The concentration of cobalt is important in determining the sensitivity of the assay (Tabor 1998); therefore, experiments were first carried out in order to determine the optimum amount of $CoCl_2$ present in the incubate that provides maximal sensitivity.

Semen was thawed at room temperature, diluted to 5 x 10^5 cells/sample and centrifuged at 2000 x g for 10 minutes. The pellet was resuspended in ice cold formaldehyde (1 % (v/v) in PBS, pH 7.4) and fixed for 15 min. After fixation, the cell solution was centrifuged at 2000 x g for 10 min, resuspended in 70 % ethanol and stored for 3 days at -20°C. Before elongation, the cells were rinsed in PBS, centrifuged at 2000 x g for 10 min and then resuspended in 50 μl of reaction buffer

(Boehringer Mannheim UK, Lewes, East Sussex, UK) containing 0.2 M potassium cacodylate, 2.5 mM Tris-HCl (pH 6.6), 0.25 mg/ml bovine serum albumin, 5 units TdT and 0.5 nM biotin-16-dUTP. The amount of $CoCl_2$ present in the incubate was from 0 to 10 mM. Samples were incubated for 30 min in the dark at 37°C, washed in PBS and resuspended in 100 µl of the fluoresceinated staining solution (0.15 M NaCl, 0.015 M sodium citrate, 2.5 µg/ml FITC-avidin, 0.1 % Triton X-100 and 5 % (w/v) non-fat dry milk). After incubating for 30 min in the dark at room temperature, samples were washed and resuspended in 1 ml PBS. Cells were further stained with propidium iodide (5 µg/ml in PBS), and 10000 analysed by flow cytometry. Control cells for each sample were treated identically except for the omission of the TdT enzyme. TdTA results were analysed by subtracting cells staining positively for FITC on the TdT-control frequency histogram from the TdT-positive histogram, yielding the number of cells possessing DNA strand breaks.

The TdTA was then performed on semen specimens obtained from 35 smokers and 35 non-smokers.

All materials were obtained from Sigma-Aldrich unless otherwise noted (Sigma-Aldrich Chemicals, Poole, Dorset, UK), with the exception of solvents, which were obtained from BDH (BDH Laboratory Supplies, Poole, Dorset, UK).

2.2.4 *Flow Cytometric Measurements*

Measurements were made using a Becton Dickinson FACS Vantage flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA) and Cell Quest software.

Immediately after staining, cells were passed through the machine and recordings started after 2 min to allow equilibration.

2.2.5 *Statistical Methods*

The data obtained using the above assays were not normally distributed (Anderson-Darling test for normality). Therefore, the non-parametric Mann-Whitney *U* was used to examine differences between samples from smoking and non-smoking donors, where a probability value of $P<0.05$ was considered significant. The statistical analyses were carried out using Minitab 11.12 (Minitab Inc., Pennsylvania, USA).

2.3 Results

2.3.1 *SCSA*

Fig. 2.3 depicts typical SCSA flow cytometric data from a non-smoker, before (fig. 2.3a) and after (fig. 2.3b) acid induced denaturation. Fig. 2.4 shows typical SCSA data from a smoker, before (fig. 2.4a) and after (fig. 2.4b) acid treatment. The scatter plots were gated to eliminate cellular debris emissions from the data acquired and show emissions at 530 nm (dsDNA) versus 630 nm (ssDNA). Frequency histograms were then produced representing the ratio of emissions at 630 : 530 nm (ssDNA : dsDNA). Prior to acid induced denaturation, both non-smoker and smoker data groups possessed similar scatter plots. Correspondingly, the frequency histograms were not distinguishable, with both displaying a single peak depicting the haploid population of cells. Following the acid treatment, a proportion of DNA in the cells denatures. This is observable on the scatter plots as a shift downwards towards the ssDNA axis and on the corresponding frequency histogram as an increase in the ssDNA : dsDNA ratio. A greater movement towards the ssDNA axis occurred to the main population of cells from the smoker samples on the scatter plots and similarly, a larger shift along the ssDNA : dsDNA axis occurred on the frequency histogram, as compared to the non-smokers' sample plots. The change in gradient of the main population of cells taken from the scatter plot before and after acid treatment was used as a measure of denaturation; as dsDNA converts to ssDNA, the slope decreases. In Table 2.1 it is demonstrated that the change in gradient is significantly greater for the sperm of smokers compared to non-smokers sperm ($P<0.02$; $n=35$). There was no

significant difference between the ratio of ssDNA : dsDNA of non-smokers' and smokers' sperm pre-acid treatment.

Fig. 2.3. Typical SCSA data from a non-smoker, before (a) and after (b) acid treatment. Panels on the left show scatter plots depicting the fluorescence emissions at 530 nm (dsDNA) versus 630 nm (ssDNA). The scatter plots were gated to remove artifactual emissions produced by cellular debris. Panels on the right show the corresponding frequency histograms depicting the fluorescence emission ratio of 630 : 530 nm (ssDNA : dsDNA).

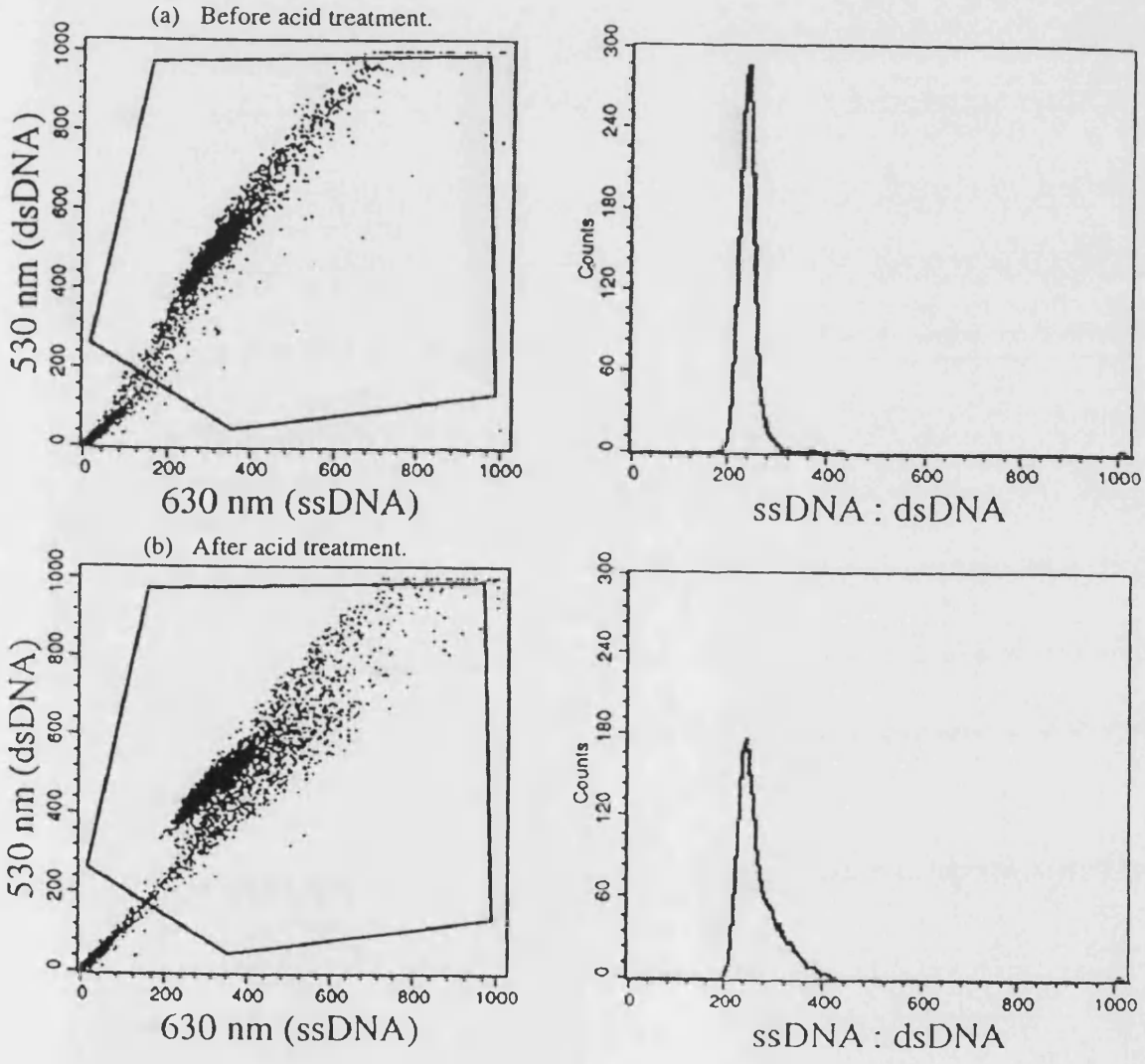


Fig. 2.4. Characteristic SCSA data from a smoker, before (a) and after (b) acid induced denaturation.

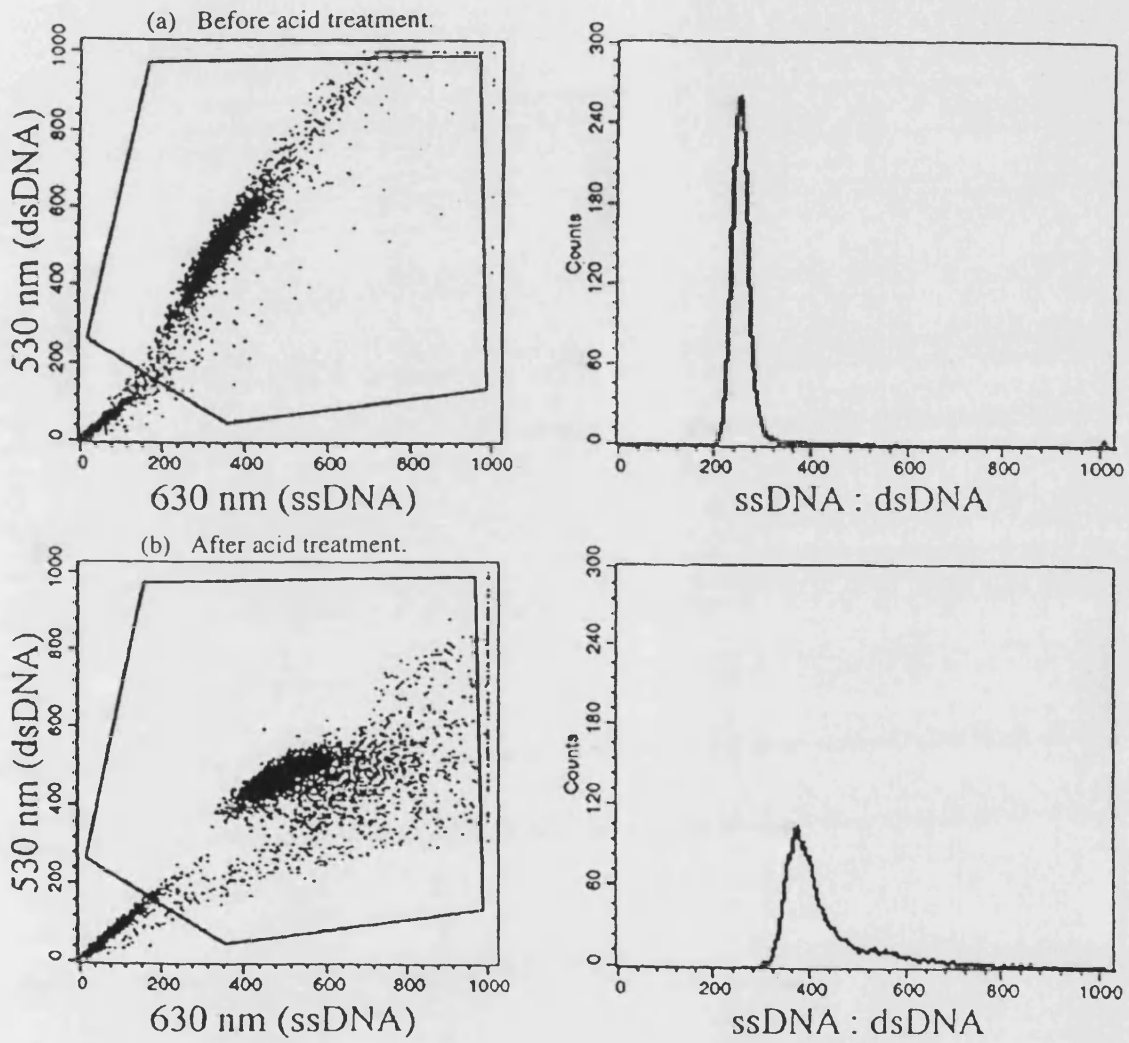


Table 2.1. Gradient of the main population of cells taken from the flow cytometric scatter plots, before and after acid induced denaturation. Values are mean gradients ± SD (n=35).

Group	Pre-acid	Post-acid
Non-smokers	52.0° ± 2.5	39.8° ± 6.7
Smokers	50.1° ± 0.7	27.9° ± 3.7*

* Comparison of the degree of DNA denaturation between smokers' and non-smokers' sperm samples post-acid treatment ($P<0.02$; n=35).

2.3.2 *TdTA*

Fig. 2.5 displays TdTA flow cytometric scatter plots produced using sperm obtained from the same specimen, in the presence of various amounts of cobalt. The optimum concentration of $CoCl_2$ in the incubate in order to provide maximal sensitivity was found to be 2.5 mM. Thus, when measuring strand breaks in sperm from smokers and non-smokers, the amount of $CoCl_2$ used during the TdT incubation was 2.5 mM.

Fig. 2.5. TdTA flow cytometric scatter plots using sperm obtained from the same sample, incubated in the presence of various amounts of cobalt. The scatter plots were gated to remove artifactual emissions produced by cellular debris. Panels on the right show frequency histograms generated by the corresponding FITC emissions. The marker (M1) on the frequency histograms depicts the percentage of cells staining positively for FITC. The incubate containing 2.5 mM $CoCl_2$ produced maximal sensitivity for the measurement of DNA strand breaks.

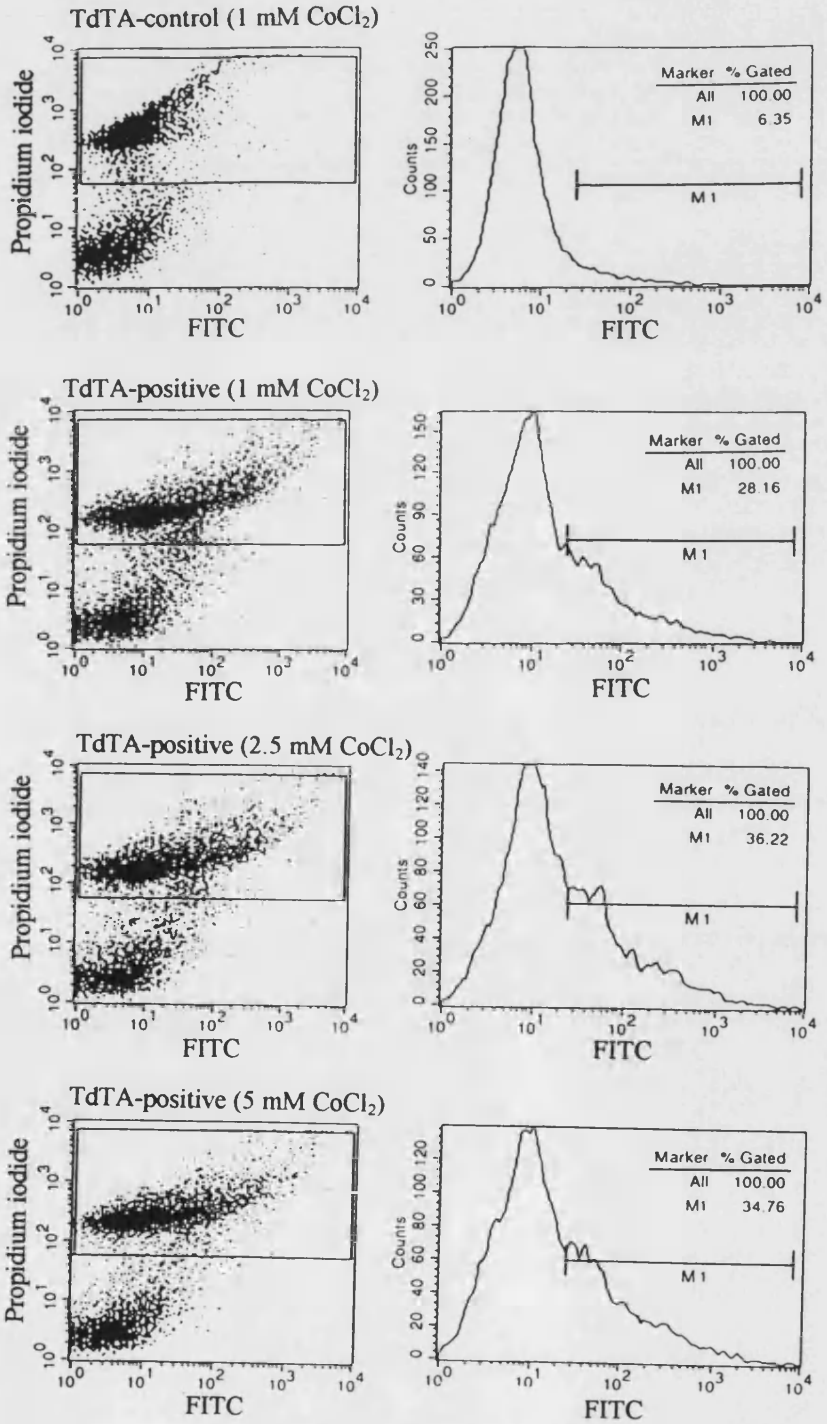


Fig. 2.6 shows typical human sperm TdTA data from a non-smoker incubated in the absence of TdT (TdTA-control; fig. 2.6a) and in the presence of TdT (TdTA-positive; fig. 2.6b). Fig. 2.7 depicts typical TdTA data from a smoker incubated in the absence of TdT (fig. 2.7a) and in the presence of TdT (fig. 2.7b). The scatter plots were again gated to eliminate cellular debris emissions from the data acquired and they represent propidium iodide versus FITC emissions. Frequency histograms were produced from the FITC emissions. The marker (M1) on the frequency histograms depicts the percentage of cells staining positively for FITC. By subtracting the number of FITC labelled cells in the TdTA-control from the TdTA-positive, the number of cells possessing strand breaks were determined (table 2.2). There was no significant difference between the TdTA-control groups of smokers versus non-smokers. The amount of strand breaks measured in smokers' sperm was found to be significantly higher compared to non-smokers' sperm ($P<0.05$; $n=35$).

Fig. 2.6. Typical TdTA data from a non-smoking donor, showing TdTA-control (a) and TdTA-positive data (b). Panels on the left show scatter plots depicting propidium iodide versus FITC fluorescence emissions. The scatter plots were gated to remove artifactual emissions produced by cellular debris. Panels on the right show frequency histograms generated by the corresponding FITC emissions. The marker (M1) on the frequency histograms depicts the percentage of cells staining positively for FITC. By subtracting the percentage of cells staining positively for FITC in the TdTA-control from those in the TdTA-positive, the percentage of cells possessing strand breaks were determined.

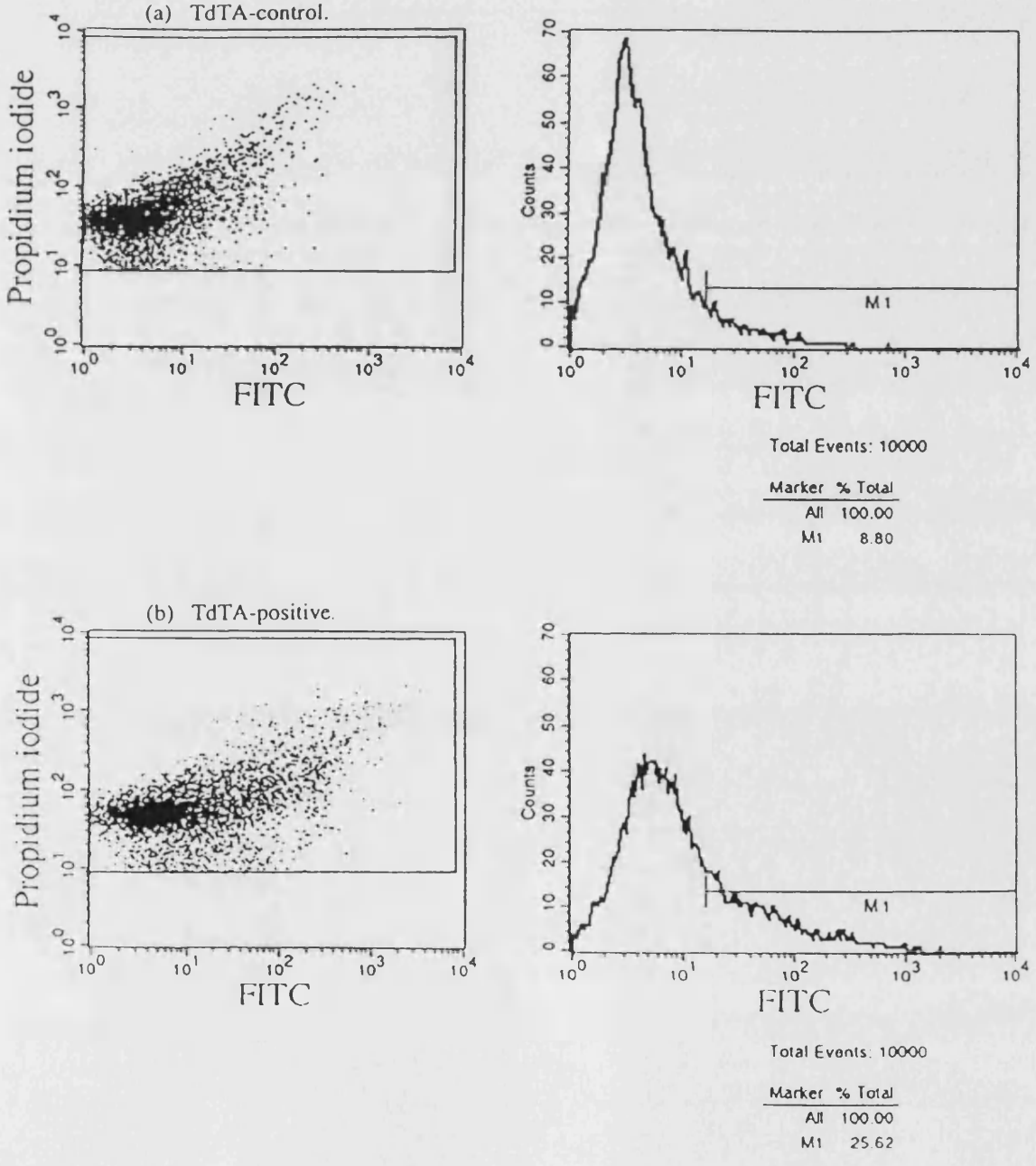
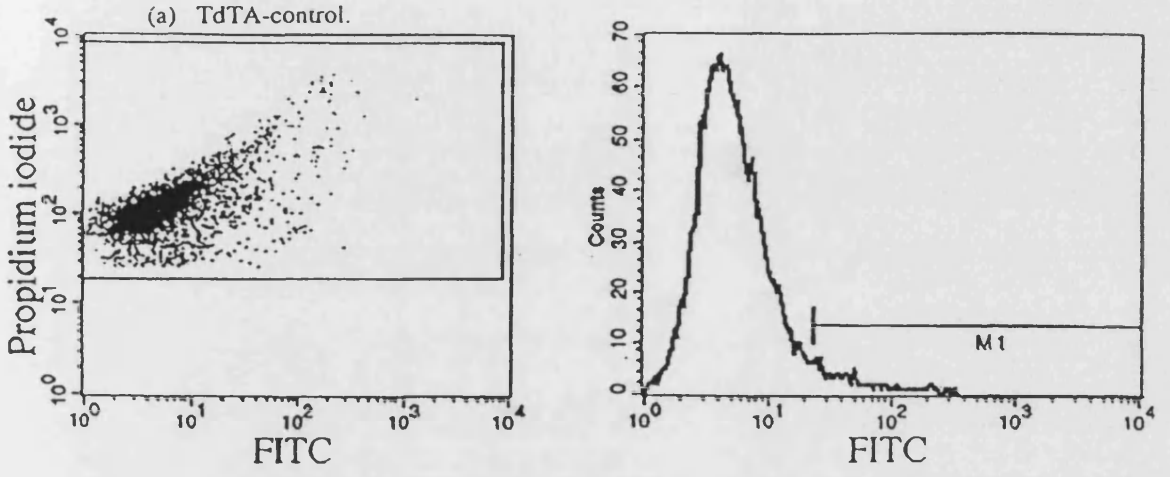


Fig. 2.7. Typical TdTA data from a smoking donor, showing TdTA-control (a) and TdTA-positive data (b).

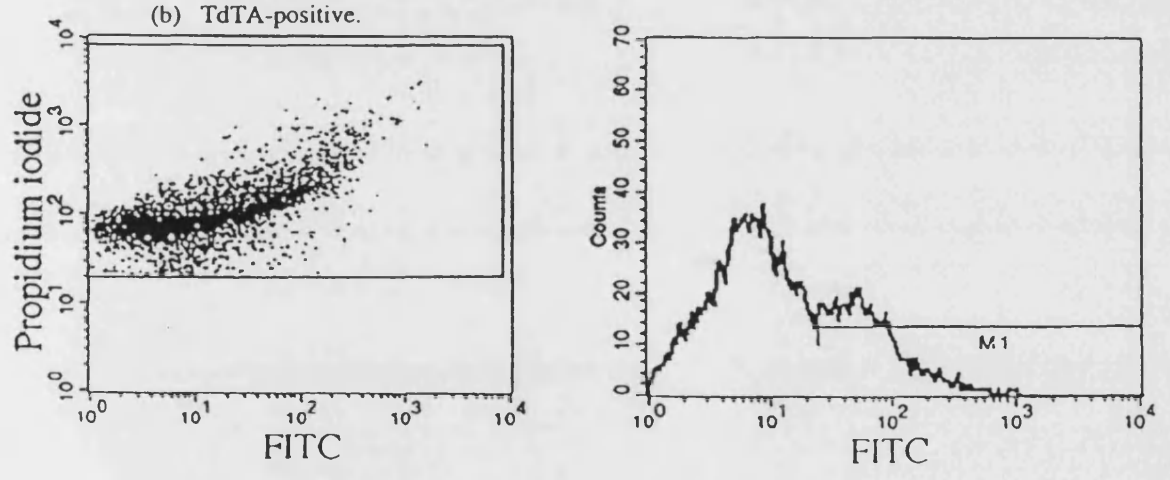
(a) TdTA-control.



Total Events: 10000

Marker	% Total
All	100.00
M1	4.89

(b) TdTA-positive.



Total Events: 10000

Marker	% Total
All	100.00
M1	31.18

Table 2.2. Percentage of sperm cells staining positively for FITC, revealing the presence of DNA strand breaks, as determined by the TdTA. Values are mean percentages ± SD (n=35).

Group	% cells staining FITC-positive in the absence of TdT enzyme (a)	% cells staining FITC-positive in the presence of TdT enzyme (b)	Difference (b-a)
Non-smokers	7.59 ± 2.12	19.57 ± 2.08	11.98 ± 3.68
Smokers	6.46 ± 2.65	25.78 ± 4.56	19.32 ± 7.68*

* Comparison of the percentage difference of DNA strand breaks in non-smokers' versus smokers' sperm ($P<0.05$; n=35).

2.4 Discussion

Some epidemiological studies have suggested that paternal cigarette smoking may predispose offspring to an increased risk of birth defects and childhood cancers (Zhang et al., 1992; Sorahan et al., 1995; 1997a; 1997b). In this study, we collected evidence to suggest that smoking adversely affects sperm nuclear material, which could potentially produce sperm DNA mutations and lead to the above observations.

Flow cytometry has evolved as a useful tool for determining the reproductive effects of exposure to environmental, occupational and therapeutic toxins. The instrument allows the rapid, sensitive, objective and multiparameter measurements of adverse reproductive interactions of chemicals and an assessment of the fertility potential of sperm (Spano and Evenson, 1993). Several determinants of sperm pathology are possible using flow cytometry. Such end points include DNA and RNA content, production of diploid sperm, altered nuclear morphology, sperm cell viability, mitochondrial function and sperm chromatin structure. In addition, precise recordings of spermatozoal DNA content allow the determination of the proportion of X- and Y-chromosome bearing cells and subsequent sorting of these subpopulations for gender pre-selection.

Results from this study demonstrate that sperm DNA from smokers is more sensitive to acid-induced denaturation and contains more strand breaks than that from non-smokers. The differences observed in this study, we suggest, are the result of oxidative stress on developing or mature sperm as a consequence of cigarette smoking. Fraga et al. (1996) found smokers' seminal plasma to contain lower levels of anti-oxidants and 50 % higher levels of 8-hydroxy-2'-deoxyguanosine, an

oxidative lesion of DNA, compared to that of non-smokers. The concept that paternal smoking caused mutations in sperm DNA potentially leading to offspring pathology was also supported in this study. Mature sperm have very little cellular repair mechanisms (Hughes et al., 1997), thus allowing oxidative DNA damage to accumulate before potential fertilisation. Additionally, Fraga et al. (1991) found that oxidative damage to sperm DNA was not necessarily associated with decreased cell motility or viability, meaning fertilisation could still be possible by a cell possessing damaged DNA.

Several tobacco related compounds have been found in smokers' seminal fluid, including nicotine and some of its metabolites, cotinine and trans-3'-hydroxycotinine (Pacifici et al., 1993; Pichini et al., 1994), precursors of the mutagenic tobacco specific nitrosamines (Hoffmann et al., 1994). Various aromatic hydrocarbons have also been discovered in seminal fluid (Notarianni et al., 1996). Rivrud (1988) determined that seminal plasma possessed an enhancing effect on the mutagenicity of benzo[a]pyrene in the Ames test, although no difference was observed between smokers' and non-smokers' samples. Additionally, the mammalian gonad possesses significant mixed function oxidase activity and is capable of metabolising a range of chemicals (Parkinson 1996). Ultimately, this may result in the activation of a chemical and formation of a reactive species capable of damaging DNA and other cellular components, that may produce diminished fertility. It is, therefore, possible that seminal plasma contains chemicals capable of causing sperm DNA damage in situ, particularly so in smokers' plasma.

We have found evidence of greater sperm DNA damage in smokers compared to non-smokers, in the form of a compromised chromatin structure when exposed to mild acid stress and more DNA strand breaks. These changes may be responsible, at least in part, to the numerous associations between smoking and male reproduction, including reduced semen quality and increased offspring pathology.

3. Smoking is associated with sperm aneuploidy

3.1 Introduction

Aneuploidy is defined as a deviation of chromosome number from an exact multiple of the haploid state (Hoffmann 1996). Cells lacking a chromosome are defined as being nullisomic, those with one chromosome are monosomic, and those with two chromosomes are disomic. Aneuploidy is one of the most serious human chromosomal abnormalities, with approximately 1 in 300 live births estimated to be aneuploid (Jacobs 1992). The actual levels of aneuploid conceptions, however, are believed to be as high as 1 in 5, supportive of the theory that the majority of aneuploid embryos spontaneously abort (Hook 1985; Obe and Anderson, 1987). Furthermore, an aneuploid individual that survives to parturition often encounters severe adverse health as a consequence, which may include physical and mental defects, as well as reduced longevity. The potential contribution of human lifestyles to the generation of aneuploidy is therefore an important area of study.

According to live birth and foetal analyses, the paternal contribution to the generation of aneuploidies varies, with proportions ranging from 100 % for XYY, 50 % for XXY, 7 % for XXX, and between 5 and 20 % for several autosomal trisomies (Bond and Chandley, 1983).

The levels of aneuploidy in sperm have been frequently investigated amongst different populations of men (Holmes and Martin, 1993; Spriggs et al., 1996; Robbins et al., 1997; Rubes et al., 1998). The occurrence of disomic sperm is around 0.05 to 0.2 % for the autosomes, and up to 0.4 % for the sex chromosomes.

3.1.1 *Induction of Sperm Aneuploidy*

Several studies have analysed the influence of social factors on the generation of sperm cell aneuploidy. Robbins and colleagues (1997) determined the potential contribution of caffeine, alcohol and smoking on sperm aneuploidy load in healthy men. Caffeine and alcohol consumption was significantly associated with increased frequencies of several aneuploidies, including both autosome and sex chromosome numerical abnormalities. Smoking was only weakly correlated with XX-18 aneuploidy, although increasing donor age was more strongly associated with the same abnormality. Rubes et al. (1998) demonstrated a significant association between cigarette consumption and the incidence of sperm disomic for chromosome Y.

3.1.2 *Lipid Peroxidation in Semen*

The determination of lipid peroxidation in sperm and seminal plasma has been performed a number of times. Suleiman et al. (1996) demonstrated higher levels of lipid peroxidation in sperm amongst asthenospermic and oligoasthenospermic men, as compared to normozoopsermic individuals. A negative correlation was also observed in the same study between spermatozoal lipid peroxidation and motility. The amount of lipid peroxides present in spermatozoa has been found to correlate with seminal plasma antioxidant activity (Smith et al., 1996), suggesting that reduced antioxidant defences may participate in the aetiology of infertility.

In the present study, we measured the percentage of sperm, obtained from smoking and non-smoking donors, that were aneuploid for chromosomes Y and 1 using fluorescence in situ hybridisation (FISH). In order to indicate whether sperm from smokers are exposed to greater levels of oxidative stress than those of non-smokers, which may result in cellular damage, lipid peroxidation was determined in both sperm and seminal plasma. In addition, the total antioxidant capacity of seminal plasma was ascertained. We report that YY1 aneuploidy is significantly higher in sperm obtained from smokers, and that the total antioxidant activity of seminal plasma is lower from that of smokers compared to non-smokers.

3.2 Methods

3.2.1 *Semen Samples*

Semen samples were obtained from patients aged between 20-30 years being screened for fertility at the Royal United Hospital, Bath, UK. All samples were collected by masturbation after 3 days of abstinence. Following liquefaction at room temperature for 1 h, semen analysis was performed by staff of the Royal United Hospital (only those classified as normozoospermic as defined by the World Health Organization (WHO 1992) were included in the study; see section 2.2.1). All samples were stored at -20°C prior to use. The smoking status of each donor was recorded and the samples used in the study characterised as either non-smokers or smokers of more than 15 cigarettes per day.

3.2.2 *Fluorescence in situ hybridisation*

Sperm aneuploidy was determined using multicolour FISH (Holmes and Martin, 1993) from semen samples donated by 20 non-smokers and 20 smokers. Aneuploidy was assessed on 10,000 cells per sample using fluorescence microscopy, by utilising DNA probes specific for chromosomes Y and 1. The Y chromosome DNA probe was labelled with fluorescein, whilst the chromosome 1 DNA probe was labelled with Texas Red, enabling distinction between the two probes emissions.

Electrostatically coated slides (BDH Laboratory Supplies, Poole, Dorset, UK) were first cleaned by immersing for 5 min each in acetone, concentrated hydrochloric acid : 95 % v/v ethanol (1 : 1) and running tap water. Slides were then stored in a coplin jar containing distilled water at 4°C until use.

Frozen semen was thawed at room temperature. Sperm cells were purified and concentrated by centrifugation through 2 ml 50 % v/v Percoll in PBS at 2000 x g for 40 min. The pellet was resuspended and washed three times in 1 ml wash buffer (10 mM Tris-HCl, 10 mM NaCl, pH 8.0), before being resuspended in 100 µl wash buffer. Spermatozoal smears were prepared on a cleaned microscope slide by placing 10 µl of the washed cell solution onto a slide and dragging the cells evenly over the slide using a coverslip. The slides were allowed to dry at room temperature for 1 h before being placed into a coplin jar containing 1 M Tris-HCl (pH 8.0) and 10 mM dithiothrietol and left at room temperature for 30 min. Slides were then removed from this solution and placed in 50 mM Tris-HCl (pH 8.0) and 10 mM 3,5-diiodosalicylic acid and left at room temperature for 2.5 h. Slides were rinsed with 2 x SSC (0.03 M sodium citrate, 0.3 M sodium chloride, pH 7.0) and air dried. The cells were then fixed in 70 %, 80 % and 100 % v/v ethanol for 2 min each and air dried. Slides were stored in a desiccator at room temperature for up to 4 weeks prior to the hybridisation procedure.

The denaturation solution (70 % v/v formamide in 2 x SSC, pH 7.0) was pre-warmed to 72°C. Slides were pre-warmed on a heating block to 37°C and placed in the denaturation solution for 8 min, processing no more than four slides at a time. After denaturation, slides were transferred quickly to 70 %, 80 % and 100 % v/v cold (-20°C) ethanol for 2 min each and air dried.

The directly-labelled satellite DNA probe (Oncor, Inc., Gaithersburg, Maryland, USA) was pre-warmed to 37°C for 5 min and vortexed gently. Probes (3µl each) were combined with 60µl hybridising solution (65% formamide in 2 x SSC; pH 7.0)

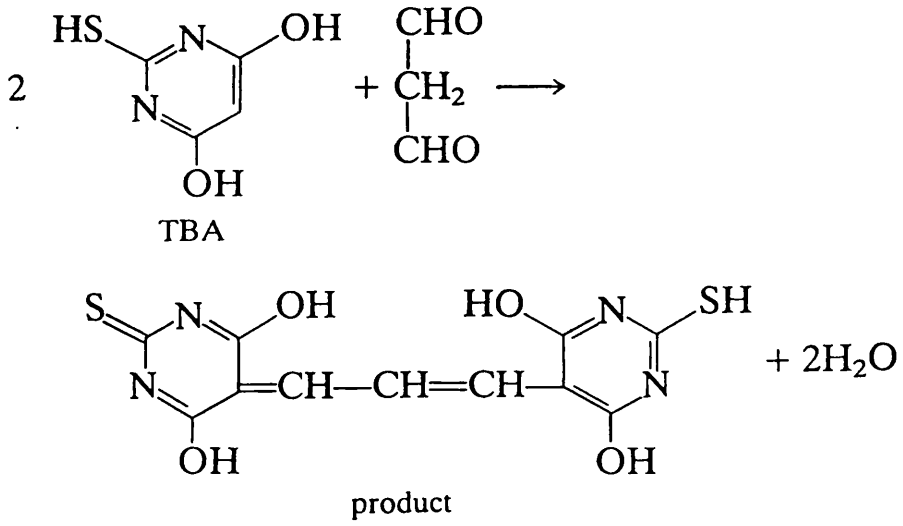
in a microcentrifuge tube and vortexed gently to mix. The probe was then denatured by heating in a 72 ± 2°C water bath for 5 min and gently vortexed to collect the contents at the bottom of the tube. The probe was placed in a 2-8°C ice bath until hybridisation. Unused probe solution was stored at -20°C and re-denatured before re-use. A 12 µl aliquot of probe solution was placed onto each slide and covered with a 22 x 22 mm glass coverslip. The perimeter was sealed with a thick layer of glass coverslip sealant (Oncor, Inc., Gaithersburg, Maryland, USA) and slides were incubated at 37°C in the dark for 3 h in a pre-warmed humidified chamber.

A wash solution containing 0.5 x SSC, pH 7.0, was pre-warmed to 72 ± 2°C in a water bath. The glass coverslip sealant was removed with forceps from the microscope slides and the coverslip carefully lifted off the slide. Slides were then immersed in the wash solution for 5 min without agitation and transferred to a coplin jar containing 1 x phosphate buffered detergent (Oncor, Inc., Gaithersburg, Maryland, USA). Counterstain was prepared by mixing 300 µl 4'6-diamidino-2-phenylindole (DAPI; 0.05 µg/ml; Oncor, Inc., Gaithersburg, Maryland, USA) with 300 µl Antifade (Oncor, Inc., Gaithersburg, Maryland, USA). Slides were removed from the phosphate buffered detergent after 10 min and excess fluid blotted from the edge, without allowing the slide surface to dry. DAPI-Antifade solution (12 µl) was added to each slide and covered with a glass coverslip (22 x 22 mm) and excess fluid blotted by pressing lightly on the slide with a tissue. Slides were evaluated using an epi-fluorescence microscope fitted with the following filters to enable visualisation of the probe and counterstain emissions:

- to view the counterstain (DAPI) signal, a 365 nm excitation and 480 nm emission filter was employed;
- to view the Texas red signal, a 540 nm excitation and 525 nm emission filter was employed;
- to view the fluorescein signal, a 490 nm excitation and 525 nm emission filter was employed.

3.2.3 *Determination of Lipid Peroxidation*

Lipid peroxidation was measured in both seminal plasma and spermatozoa by determining the production of thiobarbituric acid reactive species (TBARS; Buege and Aust, 1978). When heated, lipid peroxides decompose and produce malondialdehyde (MDA) as one of several products (Halliwell and Gutteridge, 1999). Thiobarbituric acid, or TBA, reacts with MDA to generate a coloured product that absorbs light at 535 nm, by the reaction below:



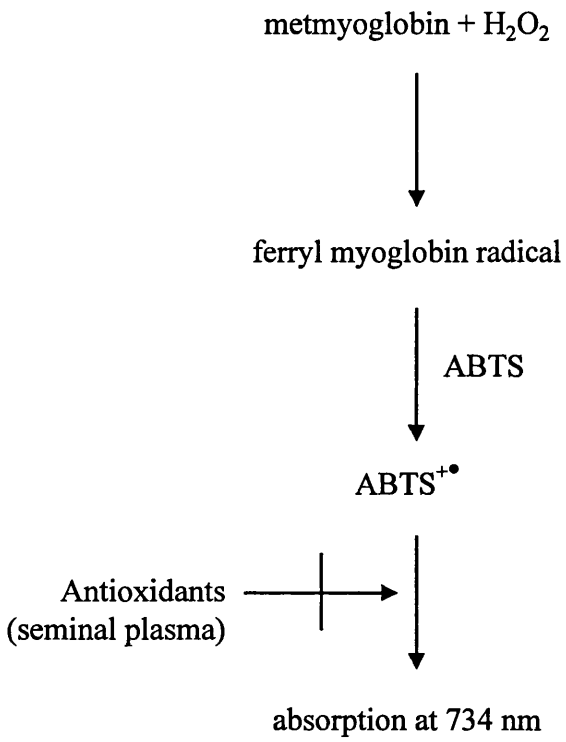
Sperm were separated from the seminal plasma by centrifugation of semen (1.5 ml) for 10 min at 2000 x g. The resulting pellet was resuspended in PBS to a concentration of 10 x 10^6 cells/ml. To 1 ml of the cell suspension and 1 ml of seminal plasma was added 2 ml thiobarbituric acid reagent (0.375 % w/v thiobarbituric acid, 15 % w/v trichloroaectic acid and 0.25 N HCl). The solutions were then incubated at 100°C for 15 min. After cooling, the precipitate was removed by centrifugation at 1000 x g for 10 min and the absorbance of the supernatant read at 535 nm against a blank containing all the reagents, replacing PBS for biological sample. The concentration of TBARS in the samples was calculated using the molar extinction coefficient of MDA-TBA adducts (1.54 x 10^5 $M^{-1}cm^{-1}$).

3.2.4 *Determination of Total Antioxidant Activity*

The antioxidant activity of biological tissues is a complex interaction between many different mechanisms, that, in combination with one another, results in the total antioxidant capacity of that matrix. Due to the complexity of the antioxidant systems, researchers have sought to produce methods that enable the measurement of the total antioxidant capacity of a sample. A method developed recently has enabled the rapid and reproducible estimation of the total antioxidant capacity of biological samples that possess only a small volume (Miller and Rice-Evans, 1996). The total antioxidant activity is determined according to the ability of plasma antioxidants to scavenge the 2'2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation ($ABTS^{+\bullet}$), inhibiting its absorption at 734 nm (Rice-Evans and Miller, 1994;

Miller and Rice-Evans, 1996). The formation and inhibition of $ABTS^{+\bullet}$ is outlined in fig. 3.1.

Fig 3.1. A flow diagram outlining the method for the determination of the total antioxidant activity in seminal plasma, as described by Miller and Rice-Evans (1996).



Metmyoglobin (MetMb) was purified prior to use. MetMb (400 μM in 10 ml PBS) was added to 10 ml potassium ferricyanide (0.0244 g in 100 ml PBS). The mixture was then applied to a 35 x 2.5 cm Sephadex G-15-120 column, pre-equilibrated with PBS, pH 7.4, and eluted with 100 ml PBS. Fractions (10 ml) were collected and the absorbance of each fraction read at 490, 560, 580 and 700 nm. The absorbance reading at 700 nm was subtracted from the readings at 490, 560 and 580 nm to correct for background absorbance. The relative proportions of MetMb were then calculated according to the equation described by Rice-Evans and Miller (1994):

$$[\text{MetMb}] = 146A_{490} - 108A_{560} + 2.1A_{580}$$

$$[\text{FerrylMb}] = -62A_{490} + 242A_{560} - 123A_{580}$$

$$[\text{MbO}_2] = 2.8A_{490} - 127A_{560} + 153A_{580}$$

Only fractions containing over 94 % MetMb were used in the assay. Purified MetMb was diluted with buffer to a concentration of 140 μM and stored at -20°C prior to use. Semen (0.5 ml) was centrifuged at 3,000 x g for 10 min. An 8.4 μl aliquot of the supernatant was added to a solution containing 2.5 μM metmyoglobin and 150 μM ABTS in phosphate buffered saline (PBS). In order to start the reaction, 375 μM hydrogen peroxide was added to the mixture, producing a final volume of 1 ml. The solution was immediately vortexed, placed in a 30°C incubator and a clock was started. After 165 s, the reaction mixture was transferred to a 1 cm cuvette and absorbance read at 734 nm. Calibrations were performed using the synthetic vitamin E analogue, Trolox, dissolved in PBS, in place of seminal plasma.

All materials were obtained from Sigma-Aldrich unless otherwise noted (Sigma-Aldrich Chemicals, Poole, Dorset, UK), with the exception of solvents, which were obtained from BDH (BDH Laboratory Supplies, Poole, Dorset, UK).

3.2.5 *Statistical Methods*

The data obtained using the above assays were normally distributed (Anderson-Darling test for normality). Therefore, the students *t* test was used to examine differences between samples from smoking and non-smoking donors, where a probability value of $P<0.05$ was considered significant. The statistical analyses were carried out using Minitab 11.12 (Minitab Inc., Pennsylvania, USA).

3.3 Results

3.3.1 *Frequency of Aneuploidy*

Smokers possessed significantly higher levels of sperm aneuploid for YY1 compared to non-smokers (0.13% and 0.08%, respectively; $P<0.05$), although no difference was seen in the frequency of sperm aneuploid for Y1-1 (0.09% and 0.11%, respectively; $P>0.05$; table 3.1). Typical results gained using the FISH procedure with human sperm is shown in fig. 3.2-3.3.

3.3.2 *Measurement of Lipid Peroxidation*

TBARS concentration was similar in both smokers' and non-smokers' seminal plasma (4.56 ± 0.34 and 5.24 ± 0.41 nmol/ml respectively; $P>0.05$) and spermatozoal fraction (0.53 ± 0.04 and 0.51 ± 0.08 nmol/ml respectively; $P>0.05$; table 3.1).

3.3.3 *Measurement of Total Antioxidant Activity*

The total antioxidant activity of seminal plasma from smokers was significantly lower than the activity determined in non-smokers' plasma (615.45 ± 140.21 and 755.76 ± 110.02 μmol/L respectively, $P<0.05$; table 3.1).

Fig. 3.2. Photomicrograph displaying sperm under fluorescence microscopy at x 600 magnification, processed using FISH. The panel displays sperm labelled with DNA probes for chromosome 1, with each cell displaying a single signal.

Fig. 3.3. Photomicrograph displaying sperm under fluorescence microscopy at x 600 magnification, processed using FISH. The panel shows sperm labelled with the Y chromosome probe. The arrow depicts an aneuploid cell, in this instance, one which possesses two Y chromosomes.

Table 3.1. Frequency of aneuploidy in human sperm obtained from smokers and non-smokers. The levels of TBARS, corresponding to lipid peroxidation, in sperm and seminal plasma, and the total antioxidant activity of seminal plasma from the two groups, is also displayed

Parameter measured	Smoker	Non-smoker
Frequency of YY1	0.13 %*	0.08 %
Frequency of Y1-1	0.09 %	0.11 %
TBARS concentration (seminal plasma)	4.56 ± 0.34 nmol/ml	5.24 ± 0.41 nmol/ml
TBARS concentration (spermatozoal)	0.53 ± 0.04 nmol/ml	0.51 ± 0.08 nmol/ml
Total antioxidant activity	615.45 ± 140.21 μmol/L*	755.76 ± 110.02 μmol/L

* Indicates a significant difference between smokers' and non-smokers' samples ($P<0.05$; *t* test; n=20).

3.4 Discussion

This study showed that sperm from donors who smoked possessed significantly more YY1 aneuploid sperm compared to sperm from non-smokers. Total antioxidant activity was significantly lower in smokers' seminal plasma compared to that of non-smokers', which may reflect greater oxidative stress in semen from smokers. No difference, however, in TBARS concentration was observed between smokers' and non-smokers' seminal plasma or spermatozoa.

Decreased total seminal plasma antioxidant activity, as demonstrated in the current study in smokers' compared to non-smokers' seminal plasma, would provide less resistance against reactive oxygen species which may in turn give rise to increased levels of cellular damage. Several studies have demonstrated an association between seminal plasma antioxidants and male fertility. Lewis et al. (1995) showed that asthenozoospermia ejaculates possessed lower seminal plasma total antioxidant activity compared to those from normozoospermic men. Men of proven fertility also show greater total antioxidant capacity compared to patients attending infertility treatment and of questionable fertility (Smith et al., 1996). In addition, DNA damage and lipid peroxidation are also associated with low seminal plasma antioxidant levels (Fraga et al. 1996; Suleiman et al., 1996). It may be of relevance that the decreased seminal plasma antioxidant capacity observed in smokers, might predispose this population to greater risk of cellular damage, that may result in reduced fertility and increased possibility of spermatozoal mutations.

Aneuploidy is a serious genetic abnormality; therefore, causative factors are of importance in order to protect human populations at risk. This study provided results

to indicate an association of smoking with the incidence of sperm aneuploidy, specifically sperm aneuploid for YY1. However, no difference was seen in the frequency of sperm aneuploid for Y1-1.

The generation of aneuploid cells usually arises as a result of non-disjunction, that is the failure of homologous chromosomes to separate at the first meiotic division or of sister chromatids to do so at the second meiotic division or mitosis (Hoffmann 1996). As a result of nondisjunction, one pole of the spindle receives both homologues or chromatids while the other receives neither. This results in the production of one daughter cell with one chromosome too many and one daughter cell with one chromosome too few. Additionally, aneuploidy may arise due to replication of an extra chromosome, loss of a chromosome during meiosis, premature centromere division and/or improper pairing of homologous chromosomes in prophase I (Hoffmann 1996).

Numerous compounds have been found to induce aneuploidy in various test systems (see Hoffmann 1996). These include pesticides, solvents and pharmaceuticals, such as anticancer and antifungal drugs. The cellular mechanisms by which agents cause aneuploidy are not clear, although aneugens are often compounds that affect multiple sites other than DNA (Oshimura and Barrett, 1986; Natarajan 1993; Spriggs et al., 1996). Compounds that alter tubule polymerisation can cause aneuploidy. For example, taxol, which enhances tubule polymerisation, and colchicine, which prevents tubule polymerisation, can induce aneuploidy in some mammalian cells. Interference with the centrioles or centromeres by agents such as ethidium bromide or diazepam, can also induce aneuploidy. Agents which affect microtubule assembly,

for instance, by interfering with microtubule-associated proteins or calmodulin, can also promote aneuploidy induction. Additionally, alkylating agents are a group of compounds that can promote the production of aneuploidy. Of the compounds present in tobacco smoke, cadmium and lead are suspected aneugens in vitro and in certain animal species in vivo (Bond and Chandley, 1983).

In conclusion, further work is required to determine if the occurrence of sperm aneuploidy in chromosomes other than those examined in this and other studies is associated with cigarette smoking, and whether it shows correlations with spermatozoal oxidative stress and/or antioxidant levels, that may indicate how aneuploidy is arising.

4. Seminal plasma reduces exogenous oxidative damage to human sperm, determined by the measurement of DNA strand breaks and lipid peroxidation.

4.1 Introduction

Oxidative stress is believed to be an important mediator of damage to spermatozoa that has been associated with various indices of cellular injury (Aitken 1995). Lipid peroxidation and DNA fragmentation have been correlated with the exposure of sperm to ROS (Chen et al., 1997; Lopes et al., 1998). In addition, excessive ROS formation by spermatozoa has been associated with decreased motility, abnormal morphology and a lowered capacity for sperm-oocyte penetration (Mann et al., 1980; Aitken and Clarkson, 1987; Aitken et al., 1989; Aitken 1994).

The preparation of sperm, prior to storage in vitro and manipulation for artificial insemination techniques, is associated with the generation of ROS and cellular damage. For example, Twigg et al. (1998) demonstrated that centrifugation of sperm is associated with both ROS formation and the production of DNA strand breaks and cellular lipid peroxidation.

In order to counteract the toxic effects of ROS, seminal plasma contains an array of antioxidant mechanisms. Semen contains high levels of ascorbate, urate and thiol groups, as well as less substantial amounts of glutathione (GSH) and α-tocopherol (Li 1975; Lewis et al., 1997; Ochsendorf et al., 1998). In addition, the antioxidant enzymes catalase, superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione reductase (GRD) have all been found in seminal plasma (Sanocka et al.,

1996; Alkan et al., 1997; Yeung et al., 1998). Spermatozoa themselves contain negligible levels of antioxidants (Mann et al., 1980; Alvarez et al., 1989), thus rendering the cells particularly reliant on their immediate environment for protection. The high levels of seminal plasma antioxidants indicate an important role in the preservation of spermatozoa. Lopes et al. (1998) demonstrated that addition of antioxidants significantly decreased the amount of spermatozoal DNA damage induced by ROS in vitro. SOD has also been found in vitro to prevent the loss of sperm motility and the induction of cellular lipid peroxidation after exposure of sperm to ROS (Kobayashi et al., 1991). Dietary ascorbic acid supplementation has been shown to produce a decrease in human sperm oxidative DNA damage (Fraga et al., 1991). Oral α-tocopherol has been associated with an increase in cell motility and reduced levels of lipid peroxidation (Geva et al., 1996; Suleiman et al., 1996). Additionally, α-tocopherol has been correlated with an improvement in the fertilisation rate of men with low fertility during an in vitro fertilisation program (Geva et al., 1996).

Washing of sperm and subsequent removal of seminal plasma removes the potential protectant capacity provided by the plasma. This situation can be exacerbated if the cells are washed and resuspended in solutions contaminated with transition metals (Halliwell and Gutteridge, 1999). Thus, the choice of solution that is used for the storage of cells in vitro should be carefully considered in order to minimise the production of cellular damage.

This study assessed the capacity of human seminal plasma to protect sperm from oxidative stress. Sperm were exposed to exogenous ROS by incubating cells in a

medium containing H_2O_2, $FeSO_4$ and ADP. Various amounts of seminal plasma were added to the incubations, and the formation of spermatozoal DNA strand breaks and lipid peroxidation determined. The purpose of this chapter is to report that seminal plasma reduces the generation of strand breaks and lipid peroxidation in human spermatozoa during exposure to exogenous oxidative stress.

4.2 Methods

4.2.1 *Semen Samples*

Semen samples were obtained from patients who being screened for fertility at the Royal United Hospital, Bath, UK. Only specimens donated by non-smokers were used in the study. All samples were collected by masturbation after 3 days of abstinence. Following liquefaction at room temperature for 1 h, semen analysis was performed by staff of the Royal United Hospital (only those classified as normozoospermic as defined by the World Health Organization (WHO 1992) were included in the study; see section 2.2.1). Specimens were then centrifuged at 2000 x g for 10 min, and the supernatant comprising the seminal plasma was removed from the cell pellet and stored at -80°C prior to use. The pellet was rinsed twice in TNE buffer (0.15 M NaCl, 0.01 Tris-HCl and Na_2EDTA, pH 7.4) before being resuspended in the same buffer and stored at -80°C prior to use. This study had the approval of the Bath District Ethics Committee.

4.2.2 *Treatment of cells*

Cells were treated with hydrogen peroxide using the method described by Chen et al. (1997). Sperm were thawed at room temperature, centrifuged at 2000 x g for 10 min and resuspended in 1 ml Biggers, Whitten and Whittingham medium (BWW; Biggers et al., 1971). From each specimen, six aliquots containing 10 x 10^6 cells were removed and placed into separate 1.5 ml vials. To five sets of cells was added 1.8 mM ADP, 0.1 mM H_2O_2 and 2.7 mM $FeSO_4$, and either 0, 10, 20, 40 or 60 % v/v seminal plasma. The cells were incubated in the seminal plasma collected from each

respective donor. The remaining set of cells was incubated in the absence of H_2O_2 and seminal plasma, in order to allow the measurement of basal levels of strand breaks or lipid peroxidation for each sample following the incubation period. The volume in each vial was made up to 0.5 ml with BWW and the cells were then incubated at 37°C for 1 h. After the incubation, the cells were centrifuged at 2000 x g for 10 min, rinsed twice in PBS and finally resuspended in 1 ml PBS before the determination of TBARS or DNA strand breaks.

4.2.3 *Determination of DNA Strand Breaks*

The TdTA allowed the measurement of DNA strand breaks by addition of biotinylated 16-dUTP to 3'-OH ends of DNA, sites of DNA breakage (Gorczyca et al., 1993; Sun et al., 1997), using the enzyme TdT. Incorporation of the nucleotide is detected using FITC-avidin and the FITC emissions then determined by fluorescence microscopy in order to ascertain the proportion of cells containing strand breaks.

The TdT assay was performed using a modified method described by Sun et al. (1997). Cells (5×10^5) were smeared onto a microscope slide and allowed to air dry. The cells were then fixed and permeabilised by submersing each slide in ice cold formaldehyde (1 % (v/v) in PBS, pH 7.4). After 15 min the cells were rinsed in PBS and placed into ice cold 70 % v/v ethanol for 1 h. The cells were then rinsed in PBS before elongation. A 50 µl aliquot of buffer containing 10 units TdT, 0.5 nmoles biotin 16-dUTP, 0.2 M potassium cacodylate, 2.5 mM Tris-HCl (pH 6.6), 2.5 mM $CoCl_2$ and 0.25 mg/ml bovine serum albumin (Roche Molecular Biochemicals, East Sussex, UK) was placed onto each slide. The samples were incubated in a humidified

chamber at 37°C for 60 min and then rinsed in PBS. The cells were then stained using 25 µl of a solution containing 0.15 M NaCl, 0.015 M sodium citrate, 2.5 µg/ml FITC-avidin, 0.1 % Triton X-100 and 5 % w/v non-fat dry milk. After incubating for 30 min in the dark at room temperature, samples were rinsed in PBS and the cells further stained with 5 µg/ml propidium iodide. Control cells for each slide were treated identically except for the omission of the TdT enzyme. A total of 1000 cells were scored per slide as either FITC-negative or -positive, yielding the proportion of cells possessing DNA strand breaks.

4.2.4 *Determination of Lipid Peroxidation*

Lipid peroxidation was measured by determining the production of TBARS, according to the method described by Buege and Aust (1978), as described in section 3.2.3.

All materials were obtained from Sigma-Aldrich unless otherwise noted (Sigma-Aldrich Chemicals, Poole, Dorset, UK), with the exception of solvents, which were obtained from BDH (BDH Laboratory Supplies, Poole, Dorset, UK).

4.2.5 *Statistical Methods*

The data obtained using the above assays were not normally distributed (Anderson-Darling test for normality). Therefore, the non-parametric Mann-Whitney *U* test was used to examine differences between the data groups where a probability value of

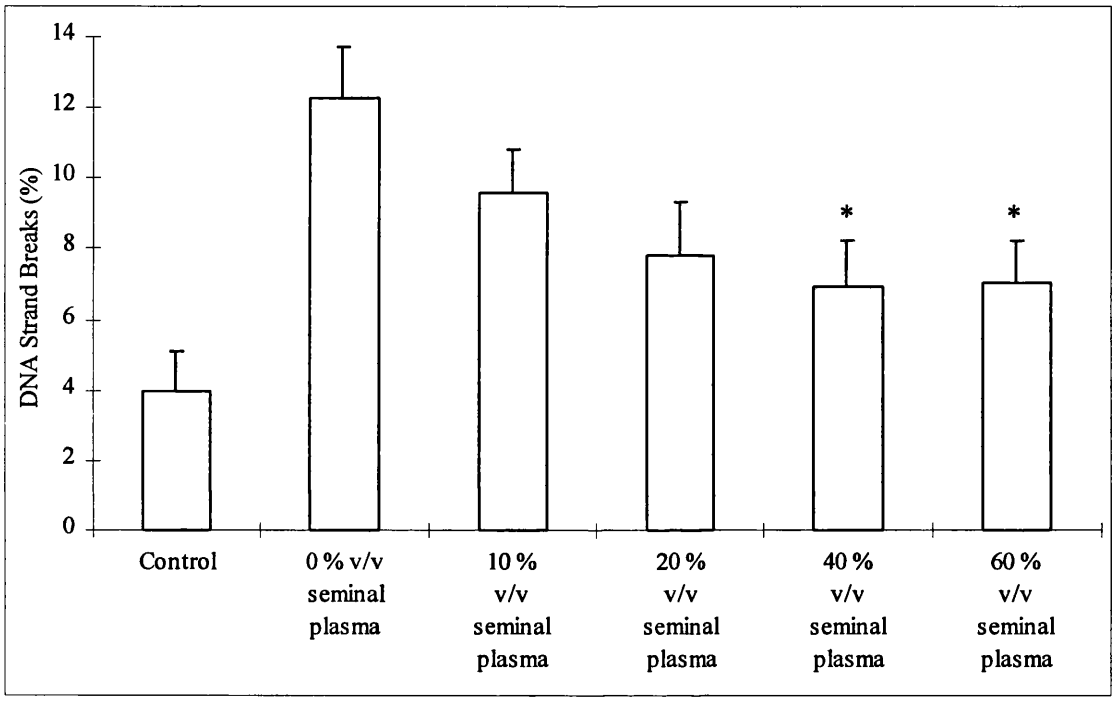
$P<0.05$ was considered significant. The statistical analyses were carried out using Minitab 11.12 (Minitab Inc., Pennsylvania, USA).

4.3 Results

4.3.1 *TdTA*

Fig. 4.1 displays the generation of DNA strand breaks in human sperm after incubation with 0.1 mM H_2O_2, 1.8 mM ADP, and 2.7 mM $FeSO_4$, in the presence of various amounts of seminal plasma. The proportion of cells possessing strand breaks after incubation in the absence of seminal plasma and hydrogen peroxide in the promoter system was 3.96 ± 1.15 %, which is similar to previously reported levels of DNA fragmentation in human sperm determined using the same method (Lopes et al., 1998). The addition of hydrogen peroxide to the system produced a 3-fold increase in the cells exhibiting strand breaks, to 12.26 ± 1.46 % ($P<0.05$). The addition of seminal plasma to the incubate produced a significant decrease in the generation of DNA strand breaks, when the amount of plasma present was 40 % v/v or greater ($P<0.05$).

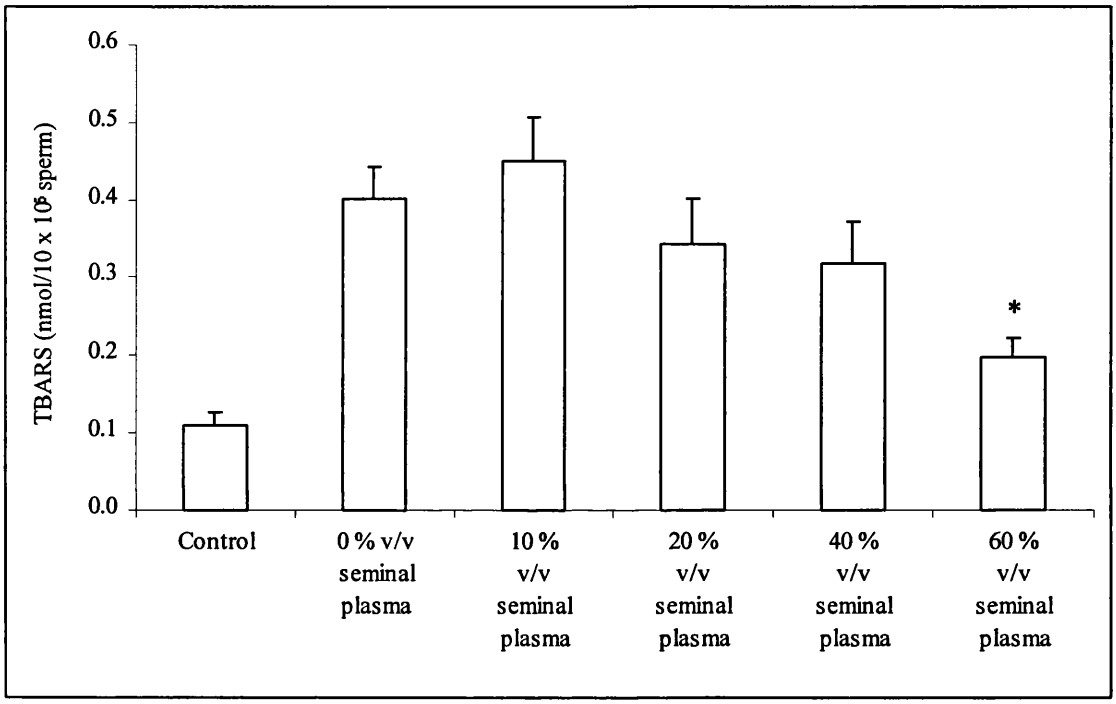
Fig. 4.1. Generation of DNA strand breaks by sperm after incubation with 1.8 mM ADP, 0.1 mM H_2O_2 and 2.7 mM $FeSO_4$ and either 0, 10, 20, 40 or 60 % v/v seminal plasma. The control bar represents sperm incubated in the absence of hydrogen peroxide and seminal plasma. Each bar displays the mean ± SEM results obtained from 6 samples. * Represents a significant difference in the production of strand breaks from the cells treated with hydrogen peroxide in the absence of seminal plasma ($P<0.05$).



4.3.2 *Lipid Peroxidation*

Fig. 4.2 displays the production of TBARS by human sperm after incubation with 0.1 mM H_2O_2, 1.8 mM ADP and 2.7 mM $FeSO_4$ in the presence of various amounts of seminal plasma. Levels of cellular TBARS in the absence of hydrogen peroxide and seminal plasma were determined to be 0.111 ± 0.016 nmol after incubation of 10 x 10^6 spermatozoa at 37°C for 1 h. The addition of hydrogen peroxide to the system produced a significant increase in the production of TBARS to 0.402 ± 0.041 nmol/10 x 10^6 spermatozoa ($P<0.05$). In fig. 4.2, it can be seen that aliquots of seminal plasma added to the incubate produced a significant decrease in cellular lipid peroxidation at a concentration of 60 % (v/v; $P<0.05$). The amount of plasma present during the incubation was also significantly associated with a lower production of TBARS (r^2 = 0.86; $P<0.05$).

Fig. 4.2. Production of thiobarbituric reactive substances (TBARS) by spermatozoa after incubation with 1.8 mM ADP, 0.1 mM H_2O_2 and 2.7 mM $FeSO_4$ and either 0, 10, 20, 40 or 60 % v/v seminal plasma. The control bar represents sperm incubated in the absence of hydrogen peroxide and seminal plasma. Each bar displays the mean ± SEM results obtained from 6 samples. * Represents a significant difference in the generation of TBARS from the cells treated with hydrogen peroxide in the absence of seminal plasma ($P<0.05$).



4.4 Discussion

The present study collected evidence to indicate that exposure of sperm to hydrogen peroxide in the presence of ferrous sulphate and ADP produces DNA strand breaks and lipid peroxidation. The addition of seminal plasma to the incubate significantly reduced the amount of oxidative damage produced in the cells.

The addition of hydrogen peroxide to many cell types generates DNA strand breaks in addition to increased levels of DNA base modification products (Halliwell and Gutteridge, 1999). However, H_2O_2 itself does not induce DNA damage. The majority of the damage in vivo is thought to be due to transition metal ion-catalysed production of the highly reactive hydroxyl radical, $OH^{\bullet}$, by the Fenton reaction. An example of Fenton chemistry in biology is the reaction of Fe^{2+} with H_2O_2, which proceeds according to the equation below:

$$Fe^{2+} + H_2O_2 \rightarrow \text{intermediate complex(es)} \rightarrow Fe(III) + OH^{\bullet} + OH^{-}$$

In concordance with the Fenton reaction, the amount of DNA damage induced in spermatozoa by H_2O_2 increases as the concentration of iron is raised in the reaction media (Chen et al., 1997).

The generation of ROS is an essential prerequisite for the normal functioning of many cells; however, excessive formation can lead to cellular pathology (Halliwell and Gutteridge, 1999). Exposure of sperm to ROS is associated with decreased fertility and the formation of lipid peroxidation and DNA damage (Mann et al., 1980; Aitken and Clarkson, 1987; Aitken et al., 1989; Aitken 1994; Chen et al., 1997; Lopes et al.,

1998). In addition, the production of lipid peroxidation in sperm due to oxidative stress has been associated with a loss of cell motility (Alvarez and Storey, 1989; Aitken and Fisher, 1994). Several studies have also demonstrated that high seminal plasma antioxidant activity correlates positively with semen quality and low levels of DNA damage and lipid peroxidation (Fraga et al., 1991; Kobayashi et al., 1991; Suleiman et al., 1996). The antioxidant capacity of sperm and the extracellular environment within which they live, therefore, would seem to be significant factors in determining the aetiology of male infertility and, potentially, the incidence of germline mutations through spermatozoal DNA damage.

Mature human spermatozoa do not possess significant DNA repair mechanisms (Chandley and Kofman-Alfaro, 1971; Hughes et al., 1997) and contain negligible levels of antioxidants (Alvarez and Storey, 1989; Aitken and Fisher, 1994). Oxidative DNA damage, consequently, has the potential to accumulate, increasing the possibility of mutagenesis during fertilisation. In addition, Fraga and colleagues (1991) demonstrated that oxidative damage to sperm DNA was not necessarily associated with decreased cell motility or viability, indicating fertilisation may still be possible by a cell that contains damaged DNA.

Reports have also suggested that paternal influences contribute more to congenital anomalies and heritable disease than maternal factors. This is thought to be due to the increased levels of cellular divisions that occur in male germ cell production compared to female gametogenesis and the lack of spermatozoal DNA repair mechanisms, resulting in an increased risk of sperm mutations (Chandley and Kofman-Alfaro, 1971; Crow 1993; Hughes et al., 1997). Factors, therefore, that

afford the protection of spermatozoa from oxidative stress are of importance. We have found evidence to suggest that seminal plasma possesses the capacity to protect sperm from the deleterious actions of oxidative attack. Seminal plasma contains high levels of antioxidants (Li 1975; Sanocka et al., 1996; Alkan et al., 1997; Lewis et al., 1997; Ochsendorf et al., 1998; Yeung et al., 1998); thus, we hypothesise that the protection of spermatozoa afforded by the plasma is as a consequence of its antioxidant properties.

The incubation of spermatozoa in the presence of hydrogen peroxide and a transition metal also generated cellular lipid peroxidation. Lipid peroxidation occurs when polyunsaturated lipids become damaged due to oxidative stress (Halliwell and Gutteridge, 1999). The sperm cell membrane contains high levels of polyunsaturated fatty acids that give the sperm considerable fluidity, necessary to allow the cell to fuse with the oocyte. This, however, renders the cell more susceptible to the generation of lipid peroxides. Lipid peroxidation in spermatozoa results in decreased membrane fluidity and a reduced capacity for successful fertilisation (Aitken 1995).

The data may be of significance in studies detecting spermatozoal damage in individuals and during in vitro fertilisation treatment. The preservation of sperm in seminal plasma during storage and cell preparation might allow for an improvement in semen quality used for artificial insemination and decrease the potential for oxidative DNA damage to arise, that may reduce germline mutations.

5. Antioxidant capacity of the epididymis

5.1 Introduction

Reactive oxygen species (ROS) are believed to be important mediators of damage to spermatozoa that have been associated with decreased motility, abnormal morphology and a lowered capacity for sperm-oocyte penetration (Mann et al., 1980; Aitken and Fisher, 1994; Aitken 1995). In order to counteract the toxic effects of ROS, seminal plasma and sperm possess an array of antioxidant mechanisms. The antioxidant enzymes catalase, superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione reductase (GRD) have all been detected in seminal plasma (Sanocka et al., 1996; Alkan et al., 1997; Yeung et al., 1998). In addition, semen contains high levels of thiol groups, ascorbic and uric acid, as well as less substantial amounts of glutathione (GSH) and vitamin E (Li 1975; Lewis et al., 1997; Ochsendorf et al., 1998). Spermatozoa themselves also possess levels of thiol groups, although substantially less than those found in seminal plasma, as well as smaller amounts of ascorbic acid, vitamin E, uric acid and GSH (Li 1975; Lewis et al., 1997; Ochsendorf et al., 1998).

5.1.1 *The Human Epididymis*

The human epididymis is a single coiled duct, measuring approximately 5 metres in length. Anatomically, the duct is arranged into three sections. From the distal to the proximal end of the epididymis, is the caput, the corpus and the cauda epididymides (Amann 1987). The final stages of sperm maturation occur in the first two segments

of the duct; whereas, the terminal section acts as a storage site for sperm prior to ejaculation. The epididymis provides an optimal environment for sperm storage and maturation (Hinton et al., 1995). The capacity of the epididymis, however, to protect sperm from oxidative attack through the local actions of antioxidants during epididymal storage has not thus far been well studied.

5.1.2 *Epididymal Antioxidants*

Yeung et al. (1998) found similar levels of SOD, GPX, GRD and catalase-like enzyme activity in human seminal plasma taken from normozoospermic and vasectomized donors (with ductal occlusion and hence no epididymal contribution to the ejaculate). Although the epididymis has been found to synthesise and secrete significant amounts of extracellular SOD, similar levels were detected in the semen of men with an intact ductal system and in that of vasectomized men (Williams et al., 1998). GSH has been located in rat, mouse and bull epididymal homogenates (Agrawal and Vanha-Perttula, 1988a; Agrawal and Vanha-Perttula, 1988b; Agrawal et al., 1989) The enzyme γ-glutamyl transpeptidase, required for the regeneration of oxidised or conjugated GSH, has also been found within the rat epididymal lumen (Hinton et al., 1991).

The mRNA distribution of various antioxidants and protective enzymes have also been investigated in the male reproductive tract of several species. Cellular, phospholipid hydroperoxide and epididymal GPX mRNA have been detected in the epididymis (Zini and Schlegel, 1997a, b). Cytoplasmic Cu,Zn- and EC-SOD mRNA have been detected in epididymal epithelia (Nonogaki et al., 1992; Zini and Schlegel,

1997a), as have low levels of catalase mRNA (Zini and Schlegel, 1996), and the gene expressing glutathione S-transferase (GST) (Hales et al.; 1980).

Although the epididymis is adapted for the storage of spermatozoa, in man, around one third of sperm found in the ejaculate emanate from extra-epididymal sites (Freund and Davis, 1969; Jouannet and David, 1978). The sperm accumulate through previous ejaculations or spontaneous leakage of sperm from the epididymis into the vas deferans (Jouannet and David, 1978), where they then survive at core body temperatures. Upon ejaculation, sperm present in the vas deferans mix with spermatozoa expelled from the epididymis. The length of time the cells remain in the vas deferans is significantly correlated with decreased cell motility, concentration and fertilising ability according to the zona-free hamster oocyte penetration assay (Jouannet and David, 1978; Lewis et al., 1984; Richardson et al., 1984).

Vasectomy is a widely used and effective method of sterilisation. The time to azoospermia is dependent on the removal of extra-epididymal sources of sperm from the reproductive tract, a period of usually 2-3 months (Freund and Davis, 1969; Marshall and Lyon, 1972; Cortes et al., 1997). Thus, ejaculates obtained from recently vasectomized men provide a useful model of the effects of extra-gonadal subsistence on sperm viability. This can assist in the understanding of epididymal function and provide information regarding detrimental effects to sperm surviving distal to the epididymis.

The aim of the present study was to assess the contribution of the human epididymis to seminal plasma antioxidant activity which may protect sperm from oxidative attack whilst stored at this site, by comparing the semen of normozoospermic and

vasectomized men. The total antioxidant activity was determined in the seminal plasma of both groups. In addition, concentrations of thiols, ascorbate and urate, individual antioxidants that are abundant in human semen, were measured. Indication of the levels of lipid peroxidation was also determined according to the amount of TBARS in seminal plasma.

In addition, the study aimed to determine whether structural changes occur in the chromatin of sperm stored in extra-epididymal sites that may adversely affect cell viability and fertilising ability, by examining sperm from vasectomized men. This was performed by staining cells with toluidine blue and recording their spectra, allowing the detection of deficient nuclear protamine disulphide crosslinking and reduced chromatin condensation (Andreetta et al., 1995). Furthermore, the SCSA (Evenson 1988), which has been correlated with exposure to several mutagenic compounds (Evenson et al., 1993a; Evenson et al., 1993b; Evenson and Jost, 1993) and fertility status (Evenson et al., 1980; Evenson et al., 1986; Sailer et al., 1995), was used to measure the susceptibility of sperm cells to acid denaturation.

The purpose of the study is to report that seminal plasma from vasectomized men contains less total antioxidant capacity, lower thiol group concentrations and higher levels of lipid peroxidation, compared to men with an intact ductal system. We also report that extra-epididymal sperm, obtained from patients after vasectomy, possess more chromatin and DNA abnormalities when compared to specimens produced by normozoospermic individuals.

5.2 Materials

5.2.1 *Semen Samples*

Semen samples were obtained from patients being screened for fertility at the Royal United Hospital, Bath, UK. All samples were collected by masturbation after 3 days of abstinence. Following liquefaction at room temperature for 1 h, semen analysis was performed by staff of the Royal United Hospital (only those classified as normozoospermic as defined by the World Health Organization (WHO 1992) were included in the study; see section 2.2.1). Semen from subjects who had undergone vasectomy were obtained 10-12 days post-operatively from the Bath Clinic, Bath, UK. Specimens were assessed by staff of the Bath Clinic and included for use in the study if the samples were azoospermic and contained $<1 \times 10^6$ leukocytes/ml. Samples were then stored at -20°C prior to use. This study had the approval of the Bath District Ethics Committee.

5.2.2 *Toluidine Blue Staining of Cells*

During human sperm cell development, somatic histones are replaced by arginine/cysteine-rich protamines, followed by chromatin stabilisation through intra- and inter-protamine crosslinking via disulphide bridges in the epididymis (Bedford, 1979; Balhorn, 1989; Ward, 1994). This produces a highly stable and condensed chromatin structure. The nucleic acid stain, toluidine blue, has been used as a predictor of semen quality and altered chromatin structure in human sperm (Barrera et al., 1993; Andreetta et al., 1995). Toluidine blue staining of spermatozoa produces mainly orthochromatic (pale blue) nuclei, whilst a small percentage of cells stain

metachromatically (violet or violet-blue) due to deficient protamine disulphide crosslinking and chromatin condensation (Andreetta et al., 1995).

Toluidine blue staining was performed according to a modified method of Andreetta et al. (1995). The procedure was carried out using semen from 18 vasectomized men and 25 men with a normal WHO semen profile. After thawing semen at room temperature, smears were made on electrostatically coated glass microscope slides (BDH Laboratory Supplies, Poole, Dorset, UK). Smears were air dried at room temperature for 1 h, fixed in absolute methanol for 5 min and air dried again. Staining was performed using 0.2 mg/ml toluidine blue (BDH Laboratory Supplies, Poole, Dorset, UK) in distilled water (pH 5.5) for 2 min. Prior to use, the toluidine blue was extracted three times with the same volume of toluene and once with chloroform to remove hydrophobic contaminants (Stockert et al., 1990). After staining, slides were washed in distilled water for 5 sec and air dried. The whole absorption spectra for the smear was then recorded using a Philips PU8710 UV/VIS spectrophotometer and PU8710/01 Falcon-Scan software (Philips, Cambridge, UK) as described previously (Gutiérrez-Gonzálvez and Stockert, 1985). The background absorption spectra of unstained cells was subtracted from the respective stained smear.

5.2.3 *SCSA*

Using flow cytometry, the SCSA measures the amount of native double stranded DNA (dsDNA; fluorescence at 515-530 nm) and denatured single stranded DNA (ssDNA; fluorescence at ≥630 nm) present in sperm before and after acid or heat

induced denaturation, using the metachromatic stain acridine orange (Evenson 1988; Evenson and Jost, 1994). Cells that possess chromatin abnormalities are more susceptible to denaturation than cells with an intact chromatin structure, and thus consequently show higher fluorescence emissions at ≥630 nm. The SCSA was performed as described in section 2.2.2, using sperm obtained from 13 vasectomized donors and 11 donors possessing a normal WHO semen profile.

5.2.4 *Determination of total antioxidant activity*

Semen was centrifuged at 3000 x g for 10 min, and the supernatant comprising the seminal plasma was removed from the cell pellet. The total antioxidant activity was determined spectrophotometrically according to the ability of seminal plasma antioxidants to scavenge the 2,2'-azinobis(3-ethylbenzothiazoline 6-sulfonate) (ABTS) radical cation, $ABTS^{+\bullet}$, inhibiting its absorption at 734 nm, as described in chapter 3, section 3.2.4. In order to study whether the antioxidant capacity varied over a period of time, determinations of the total antioxidant activity in seminal plasma was carried out in samples donated every two weeks by the same individual over a period of eight weeks.

5.2.5 *Determination of total thiol concentration*

Total plasma thiol concentration was determined according to the method described by Hu (1994). Semen (1 ml) was centrifuged at 3000 x g for 10 min. A 200 µl aliquot of the supernatant was mixed with 600 µl 0.25 M Tris base containing 0.20 mM Na_2ETDA, pH 8.2, 40 µl 10 mM 2,2-dithiobisnitrobenzoic acid and 3.16 ml

absolute methanol. The solution was vortex mixed, left to stand at room temperature for 20 min, and then centrifuged at 3000 x g for 10 min. The absorbance of the clear supernatant was measured at 412 nm and subtracted from a blank containing distilled water in place of plasma. Total thiol concentration was then calculated using a standard curve constructed using reduced glutathione (GSH) in distilled water in place of plasma.

5.2.6 *Determination of TBARS concentration*

Semen was centrifuged at 3000 x g for 10 min, and the supernatant comprising the seminal plasma removed from the cell pellet. Lipid peroxidation was then determined in the seminal plasma according to the concentration of TBARS in the seminal plasma, as described in chapter 3, section 3.2.3.

5.2.7 *Determination of ascorbic acid and uric acid concentration*

Total plasma ascorbic and uric acid concentrations were determined according to a modified method of Pappa-Louisi and Pascalidou (1998), using HPLC with UV detection. The liquid chromatography system consisted of a Jasco PU-980 pump, a Jasco 851-AS autosampler fitted with a 50 µl injection loop, a Hichrom 5 µm 150 x 4.6 mm C18 column (Hichrom Limited, Reading, UK) and a Jasco UV-975 detector set at 280 nm (Jasco UK, Great Dunmow, UK). The mobile phase consisted of 0.05 M disodium hydrogen phosphate buffer containing 2 mM Na_2EDTA and 5 mM cetyltrimethylammonium bromide, adjusted to pH 4.0 using orthophosphoric acid. The mobile phase was filtered through a 0.45 µm filter under vacuum and degassed

using helium before use. All separations were carried out at ambient room temperature, using a flow rate of 0.8 ml/min.

Semen (1 ml) was centrifuged at 3000 x g for 10 min. A 300 µl aliquot of the supernatant was admixed with an equal volume of cold 10 % w/v metaphosphoric acid containing 2 mM Na_2EDTA. A 10 µl aliquot of 3,4-dihydroxybenzylamine (5 mg/ml) was added to the acidified plasma solution, which acted as an internal standard for the quantification of ascorbic acid. Unlike ascorbic acid, uric acid is stable in sample and standard solutions and was quantified using an external uric acid calibration. The mixture was vortexed and centrifuged at 3000 x g for 10 min at 4°C. A 100 µl aliquot of the supernatant was added to 50 µl 0.5 M Tris buffer containing 10 mM dithiothreitol, pH 9.0, in order to reduce oxidised ascorbic acid (dehydro-L-ascorbic acid) to ascorbic acid. After 5 min at 25°C, the reaction was quenched by the addition of 50 µl 0.2 M H_2SO_4. The volume was made up to 0.5 ml with 0.05 M disodium hydrogen phosphate buffer containing 2 mM Na_2EDTA, pH 4.0, and filtered through a disposable 0.2 µm filter into a capped vial. Prior to injection, the sample was stored on ice in the dark. An injection volume of 50 µl was used for the analyses. All standards were prepared in mobile phase without the addition of cetyltrimethylammonium bromide.

All materials were obtained from Sigma-Aldrich unless otherwise noted (Sigma-Aldrich Chemicals, Poole, Dorset, UK), with the exception of solvents, which were obtained from BDH (BDH Laboratory Supplies, Poole, Dorset, UK).

5.2.8 *Statistical analysis*

An Anderson-Darling test for normality was carried out on each data group to be examined statistically. Depending on the outcome, either an unpaired *t* test or a Mann-Whitney *U* test was employed to test differences between samples from normozoospermic donors and from vasectomized donors. A P value of < 0.05 was considered statistically significant. All statistical analyses were carried out using Minitab 11.12 (Minitab Inc., PA, USA).

5.3 Results

5.3.1 *Profile of Semen Obtained from the Donors used in the Study*

Table 5.1 shows the semen characteristics of the ejaculates used in the study. The data concurs with that reported previously (Lewis *et al.*, 1984; Richardson *et al.*, 1984), in that vasectomy results in a reduction in sperm cell concentration and motility ($P<0.05$).

Table 5.1. Semen characteristics of normozoospermic and post-vasectomy ejaculates, obtained for use in the study. Values are mean ± SD.

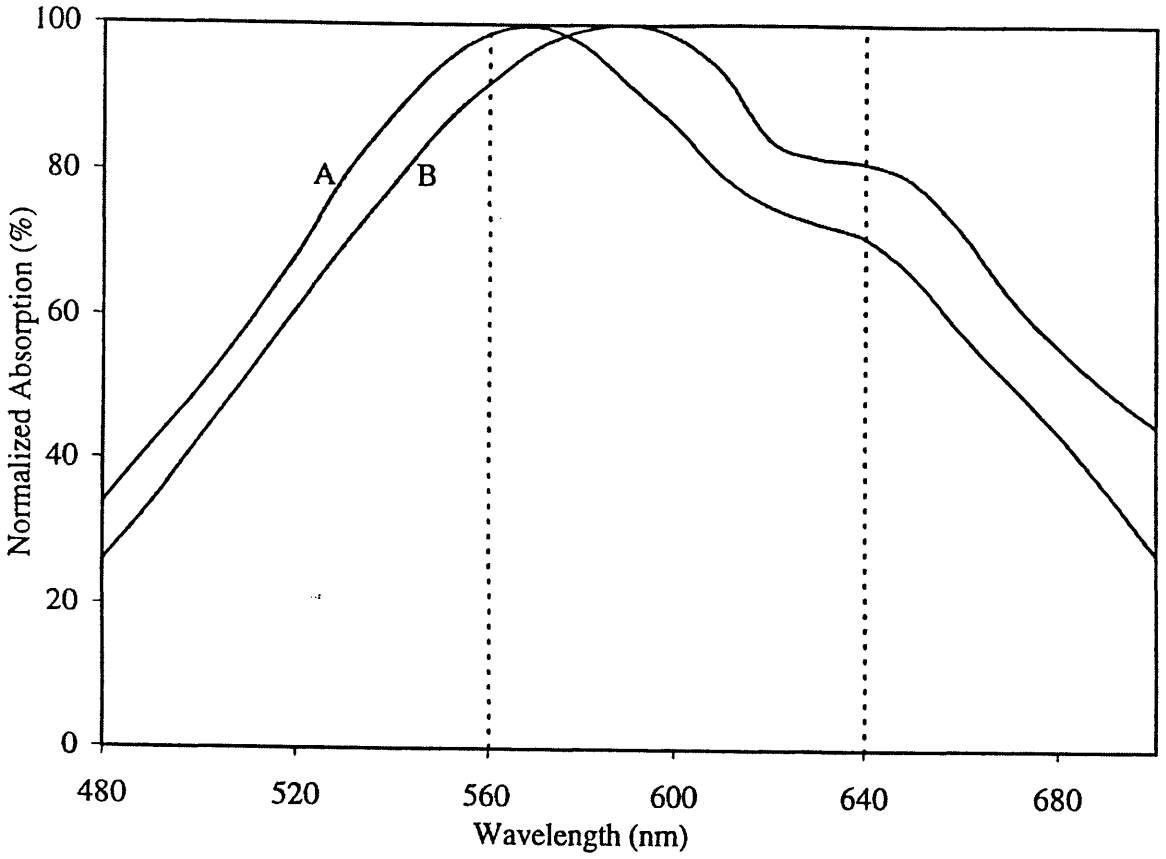
Donor group	Volume (ml)	Sperm count (x 10^6/ml)	Motility (% forward progression)	Normal forms (%)
Post-vasectomy (n=20)	3.5 ± 1.1	23.2 ± 10.3*	18.2 ± 5.3*	50.3 ± 12.4
Normozoospermic (n=29)	3.3 ± 0.9	97.1 ± 38.9	63.2 ± 9.7	57.5 ± 10.3

* Represents a significant difference from the normozoospermic donors' sperm ($P<0.05$; *t* test).

5.3.2 *Toluidine Blue Staining of Cells*

A typical normalised absorption spectra for human sperm stained with toluidine blue obtained from a normozoospermic donor and from a donor post-vasectomy is shown in fig. 5.1. Normozoospermic sperm smears produced an absorption maxima at 585-600 nm, with a shoulder at 635-645 nm. Extra-epididymal sperm smears produced a shift in the absorption spectra compared to normozoospermic sperm spectra, with both absorption maxima and shoulder being at shorter wavelengths, 565-580 and 625-635 nm respectively. The spectra produced were quantified using the orthochromatic/metachromatic (OM) ratio, or the ratio of absorption at 640 nm/560 nm, as described by Andreetta et al. (1995).

Fig. 5.1. Normalised absorption spectra of methanol fixed human sperm smears stained with the nucleic acid dye toluidine blue. Curve A represents typical spectra for a vasectomized patient and curve B for a normozoospermic donor. Vertical dotted lines indicate the absorption values at 560 and 640 nm, which were used to calculate the orthochromatic/metachromatic ratio (OM ratio; 640/560 nm) for each sample.



The mean ± SD absorption at 560 nm and 640 nm, and the OM ratio of sperm from 25 normozoospermic donors and 18 vasectomized donors, are displayed in table 5.2. Normozoospermic donors' sperm smears produced a significantly higher OM ratio when compared to extra-epididymal sperm smears ($P<0.01$; *t* test). The absorption spectra were less intense for the vasectomized group compared to the normozoospermic group due to lower cell concentrations. Hence, reduced absorption is observed at 560 nm and 640 nm. The wavelength absorption peaks found using spectral analysis of nucleic acid-dye complexes, however, have been shown to be independent of cell concentration (Gutiérrez-Gonzálvez and Stockert, 1985).

Table 5.2. Toluidine blue staining characteristics of human sperm from normozoospermic donors and donors post-vasectomy, evaluated from their spectral recordings. The ortho-/metachromatic (OM) ratio represents the ratio of absorption at 640 nm/560 nm. Values are mean ± SD.

Donor group	Absorption at 560 nm	Absorption at 640 nm	OM ratio
Post-vasectomy (n=18)	0.079 ± 0.039	0.054 ± 0.034	0.657 ± 0.151*
Normozoospermic (n=25)	0.125 ± 0.188	0.117 ± 0.193	0.956 ± 0.201

* Represents a significant difference from the normozoospermic donors' sperm ($P<0.01$; *t* test).

5.3.3 *SCSA*

Fig. 5.2 depicts typical SCSA flow cytometric scatter plots from a normozoospermic donor, before (Fig. 5.2a) and after (Fig. 5.2b) acid induced denaturation. Fig. 5.3 shows typical SCSA scatter plots from a vasectomized donor, before (Fig. 5.3a) and after (Fig. 5.3b) acid treatment. The scatter plots were gated to eliminate cellular debris emissions from the data acquired and show emissions at 530 nm (ds or native DNA) versus 630 nm (ss or denatured DNA). Before acid induced denaturation, the scatter plots show a central cluster of cells, depicting the haploid population of sperm. Following the acid treatment, sperm that show increased sensitivity to acid denaturation move out of the main population of cells. The proportion of cells outside of the main population was defined by the triangular regions depicted on the scatter plots in fig. 5.2 and 5.3. Note that the position of the main cluster of cells from the normozoospermic donor changed little after acid treatment, indicating that the majority of the cells' DNA was resistant to the acid treatment. In contrast, the vasectomized donors' sperm produce a marked shift in the acridine orange fluorescence emissions after acid treatment towards the 630 nm axis. In table 5.3, it can be seen that, before acid treatment, significantly more sperm were positioned outside of the main population of cells from the samples acquired post vasectomy compared to the normozoospermic samples (8.00 ± 8.19 and 0.53 ± 0.54 % respectively; $P<0.05$; *t* test). Concurrently, after acid treatment, there was significantly more sperm outside of the main population of cells from the samples acquired post vasectomy compared to the normozoospermic samples (15.86 ± 10.72 % and 9.35 ± 7.25 % respectively; $P<0.05$; *t* test).

Fig. 5.2. Typical SCSA data from a normozoospermic donor, before (a) and after (b) acid treatment. Panels show scatter plots depicting the fluorescence emissions of 5000 cells at 530 nm (ds or native DNA) versus 630 nm (ss or denatured DNA), with each dot representing the emission from a single cell. The cells positioned outside of the main population of normal sperm were defined by the triangular regions depicted on the scatter plots. The plots were gated to remove artifactual emissions produced by cellular debris.

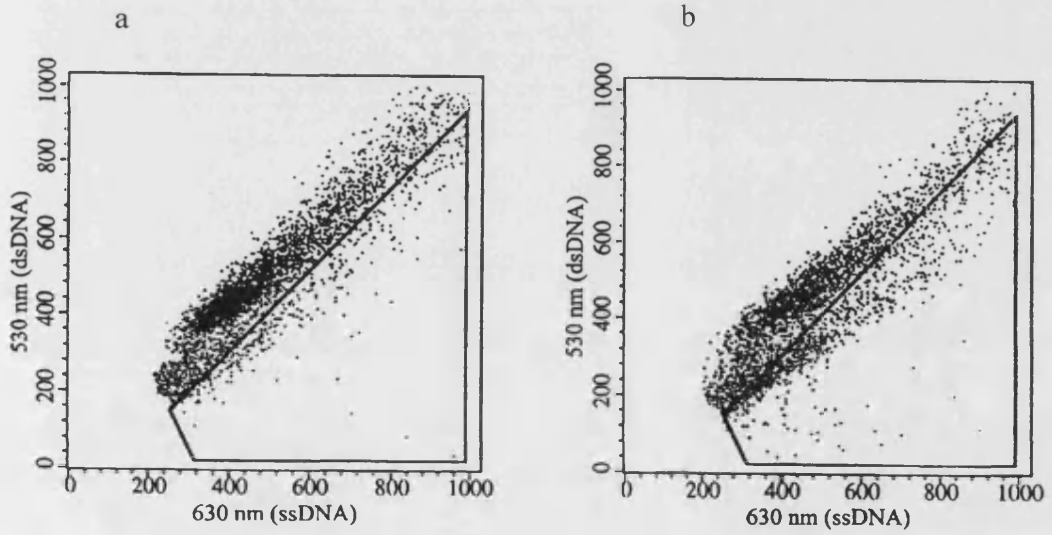


Fig. 5.3. Characteristic SCSA data from a donor after vasectomy, before (a) and after (b) acid induced denaturation.

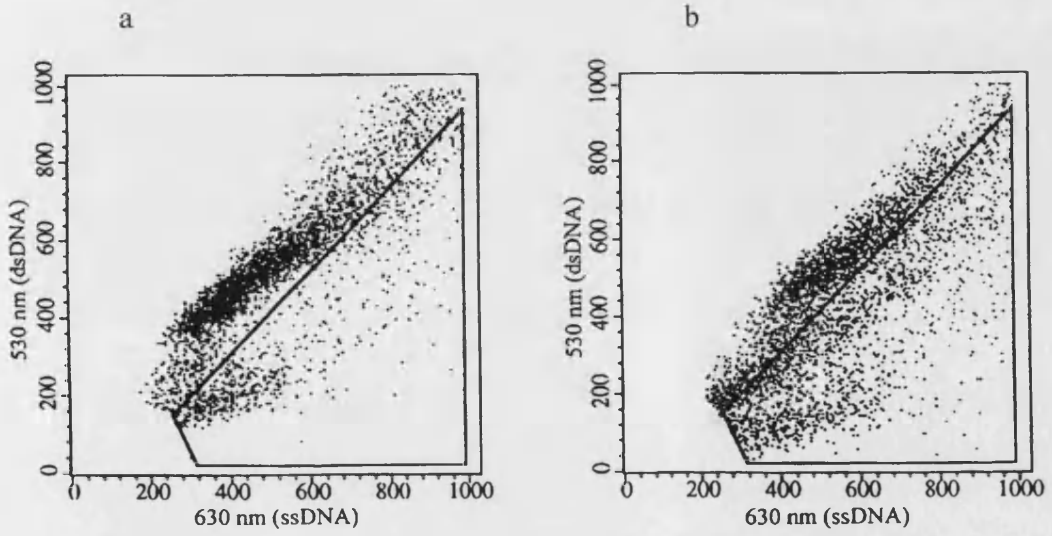


Table 5.3. SCSA data of human sperm obtained from normozoospermic and vasectomized donors. The data shows the percentage of sperm that are outside the main population of cells before and after acid treatment, determined by the scatter plots depicting acridine orange fluorescence emissions at 530 nm (ds or native DNA) and 630 nm (ss or denatured DNA). Values are mean percentages ± SD.

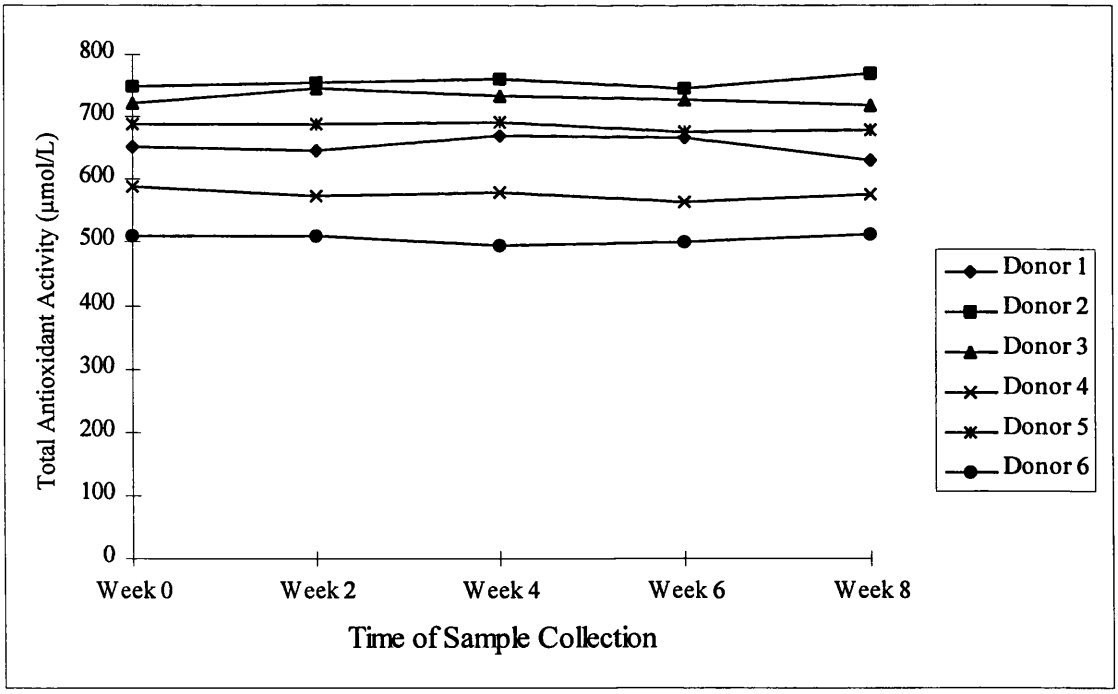
Donor group	Before acid	After acid
Post-vasectomy (n=13)	8.00 ± 8.19 %*	15.86 ± 10.72 %*
Normozoospermic (n=11)	0.53 ± 0.54 %	9.35 ± 7.25 %

* Represents a significant difference from the normozoospermic donors' sperm ($P<0.05$; *t* test).

5.3.4 *Measurement of total antioxidant capacity*

The total antioxidant capacity of seminal plasma in normozoospermic samples ranged from 473.69 to 887.84 μmol/L, and it ranged from 316.84 to 825.83 μmol/L in samples acquired from men with ductal occlusion (table 5.4). The normozoospermic group possessed significantly higher total antioxidant activity than that obtained using plasma from vasectomized individuals (680.17 ± 103.94 μmol/L and 606.69 ± 104.86 μmol/L respectively; $P<0.02$; *t* test). The total antioxidant capacity did not significantly vary in samples obtained from six normozoospermic and four vasectomized individuals every two weeks over a period of two months (fig. 5.4).

Fig. 5.4. Total antioxidant activity of seminal plasma, determined in the same six normozoospermic donors at two week intervals over a period of eight weeks.



5.3.5 *Measurement of thiol concentration*

Plasma thiol content ranged from 76.93 to 261.71 μmol/L in normozoospermic men and 59.91 to 227.68 μmol/L in vasectomized men (table 5.4). Thiol concentration was significantly higher in the plasma of normozoospermic men compared to that of vasectomized individuals (128.60 ±45.81 and 101.55 ± 40.09 μmol/L respectively; $P<0.05$; Mann-Whitney *U* test).

5.3.6 *Measurement of TBARS concentration*

Plasma levels of TBARS ranged from 2.71 to 9.11 nmol/ml in the normozoospermic group and 4.59 to 10.00 nmol/ml in the vasectomized group (table 5.4). The concentration of TBARS were significantly higher in the seminal plasma of samples obtained from vasectomized donors compared to normozoospermic donors (7.98 ± 1.36 and 5.66 ± 1.38 nmol/ml respectively; $P<0.001$; *t* test).

5.3.7 *Measurement of ascorbic acid and uric acid concentration*

Fig. 5.5 displays a typical chromatogram of a seminal plasma extract, performed as described under materials and methods. Under the conditions employed, all peaks of interest were well resolved from interfering peaks and eluted in less than 12 min. Total seminal plasma ascorbic acid content ranged from 214.81 to 523.40 μmol/L in all individuals (table 5.4). No significant difference in plasma ascorbic acid content was found between normozoospermic and vasectomized men (384.88 ± 122.23 and 396.48 ± 87.20 μmol/L respectively; $P>0.05$; *t* test). Levels of uric acid in seminal plasma ranged from 119.73 to 312.02 μmol/L in all individuals (table 5.4). There was

also no significant difference in uric acid content between the normozoospermic and post-vasectomy data groups (197.62 ± 66.73 and 189.51 ± 53.34 μmol/L respectively; $P>0.05$; *t* test).

Fig. 5.5. Typical chromatogram of a seminal plasma extract executed as described under materials and methods, to allow the measurement of ascorbic and uric acid. The mobile phase (pH 4.0) consisting of 0.05 M disodium hydrogen phosphate, 2 mM Na_2EDTA and 5 mM cetyltrimethylammonium bromide, produced retention times of 6.69 min for uric acid, 9.15 min for ascorbic acid, and 3.20 min for the ascorbate internal standard, 3,4-dihydroxybenzylamine.

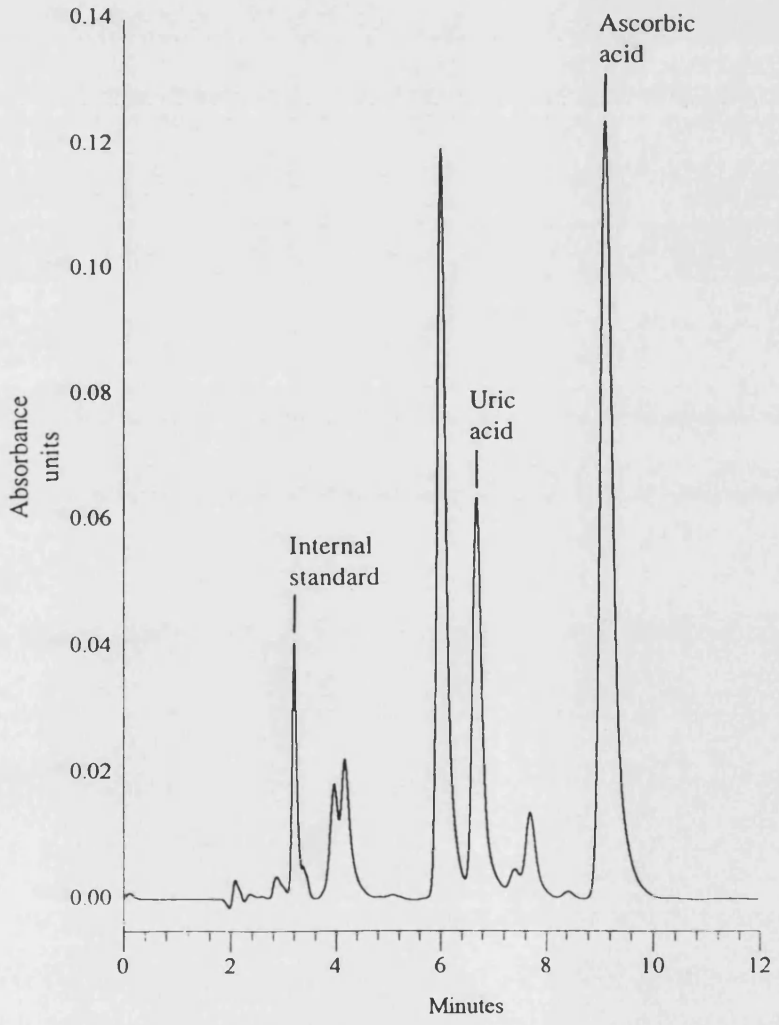


Table 5.4. Concentrations of antioxidants and malondialdehyde in the seminal plasma of vasectomized and normozoospermic men. Values shown are mean ± SD.

Parameter measured	Post-vasectomy donor (n)	Normozoospermic donor (n)
Total antioxidant capacity (μmol/L)	606.69 ± 104.86 (n=23)*	680.17 ± 103.94 (n=23)
Thiol groups (μmol/L)	101.55 ± 40.09 (n=16)*	128.60 ±45.81 (n=16)
Ascorbic acid (μmol/L)	396.48 ± 87.20 (n=18)	384.88 ± 122.23 (n=18)
Uric acid (μmol/L)	189.51 ± 53.34 (n=18)	197.62 ± 66.73 (n=18)
TBARS (nmol/ml)	7.98 ± 1.36 (n=19)***	5.66 ± 1.38 (n=21)

* Represents a significant difference from the normozoospermic donors' sperm (*P<0.05; ***P<0.001).

5.4 Discussion

This study investigated whether the human epididymis is a region-specific source of antioxidants that are measurable in the ejaculate by comparing the semen of normozoospermic and vasectomized men. Total antioxidant activity was measured, as were concentrations of the individual antioxidants, ascorbate, urate and thiol groups. Concentration of TBARS, breakdown products of lipid peroxidation, were also determined. Additionally, the study used the SCSA to detect the sensitivity of human extra-epididymal sperm DNA to acid induced denaturation. Also, toluidine blue staining of the DNA in such cells allowed the assessment of protamine disulphide crosslinking and chromatin condensation.

The study is the first, to our knowledge, to demonstrate that seminal plasma from men with an intact ductal system possesses higher total antioxidant capacity and greater thiol group concentrations, when compared with plasma from vasectomized individuals. Concentrations of lipid peroxidation were lower in the seminal plasma of normozoospermic men compared to specimens from post-vasectomy donors, which may be due in part, to the greater total antioxidant capacity that was demonstrated. Furthermore, the results indicate that sperm surviving extra-epididymally in the male reproductive tract, after vasectomy, possess chromatin abnormalities, namely reduced protamine disulphide crosslinking, deficient chromatin condensation, and increased susceptibility to acid-induced denaturation.

The generation of ROS is an essential prerequisite for the normal functioning of many cells; however, excessive formation can lead to cellular pathology. Over-exposure of sperm to ROS is associated with decreased motility, abnormal morphology and a

lowered capacity for oocyte penetration. Several studies have also demonstrated that high levels of certain antioxidants correlate positively with semen quality (Fraga et al., 1991; Fraga et al., 1996; Kobayashi et al., 1991; Suleiman et al., 1996). The antioxidant capacity of sperm and the environs within which they live would seem, therefore, to be significant factors in determining the aetiology of male infertility.

Ascorbate, urate and thiols are the major individual antioxidants present in human semen (Lewis et al., 1997), and were therefore selected for measurement in this study. Thiol content in normozoospermic semen was significantly higher than in samples obtained from vasectomized men, suggesting the accumulation of thiol containing compounds such as GSH in the epididymal lumen. Ascorbic acid is present in blood plasma at levels of around 40 μmol/L, considerably lower than the seminal plasma content found in this and other studies (Fraga et al., 1991; Thiele et al., 1995; Lewis et al., 1997), suggesting that ascorbate has an important physiological role in the male reproductive tract. Previously, ascorbate has been shown to be negatively correlated with sperm oxidative DNA damage (Fraga et al., 1991), seminal plasma and spermatozoal ROS production (Thiele et al., 1995; Lewis et al., 1997), and abnormal sperm morphology (Thiele et al., 1995). Uric acid is an important antioxidant in blood plasma, where it is found at concentrations of approximately 200 μmol/L (Halliwell and Gutteridge, 1986), comparable levels to those detected in seminal plasma in the current study. However, as with ascorbic acid, uric acid was present at similar levels in both normozoospermic and vasectomized men. Ascorbate and urate do not, therefore, appear to be key antioxidants in the epididymis.

The total antioxidant capacity of seminal plasma, calculated in this study according to the ability of antioxidants to scavenge the radical cation, $ABTS^{+\bullet}$, was determined to be 680.17 ± 103.94 μmol/L for the normozoospermic group. This figure is under half the total antioxidant capacity determined using the same method for adult human blood plasma (mean 1.46 ± 0.14 mM; Rice-Evans and Miller, 1994), but it was significantly higher activity than that found in the plasma of men with ductal occlusion. The antioxidant capacity of an individual did not change significantly when samples were collected from the same donor every two weeks over an eight week period. Thus, it would seem that, over short periods at least, the total seminal plasma antioxidant activity is reasonably stable.

Using the antioxidant data obtained from vasectomized and normozoospermic men, the antioxidant capacity of the epididymis can be estimated by taking into account the proportion of the ejaculation volume that is contributed by the epididymis. Jouannet and David (1978) estimate that the epididymis voids approximately 0.7 ml in the average ejaculate, or around seventeen percent. The difference in antioxidant capacity determined between individuals with an intact ductal system and vasectomized men in the present study is 73. 48 μmol/L (680.17 – 606.69 μmol/L). If we assume that the volume the epididymis contributes to the ejaculation is 0.7 ml, then an estimated antioxidant capacity of the epididymal fluid is 51.44 nmol.

The elevated levels of TBARS found in the seminal plasma of vasectomized individuals concur with the lower antioxidant capacity that was demonstrated in this group, in that reduced antioxidant levels would provide less protection against the deleterious effects of ROS, such as generation of lipid peroxidation. Overall, the data

appears to indicate that certain antioxidants, such as thiol containing compounds but not urate and ascorbate, accumulate in the epididymal lumen, where they may then protect against the adverse effects of ROS.

Toluidine blue staining of spermatozoa demonstrated that when stored distal to the epididymis, the cells possess lower protamine disulphide crosslinking and reduced chromatin condensation compared to normozoospermic cells. These abnormalities have previously been correlated with reduced fertility of human sperm (Barrera et al., 1993; Andreetta et al., 1995).

The SCSA produced considerable differences between the relatively homogeneous acridine orange staining pattern of normozoospermic cells and the heterogeneous staining pattern of sperm from vasectomized donors. Both before and after acid treatment, significantly more extra-epididymal sperm were situated outside of the main population of cells compared to normozoospermic sperm, indicating damaged chromatin and DNA structure. Increased sensitivity to acid induced denaturation in the SCSA has previously been shown to be predictive of high levels of chromosomal breaks (Estop et al., 1993) and DNA strand breaks (Sailer et al., 1995). The results also concur with the toluidine blue data, in that infertile specimens have been shown to be more sensitive to acid- or heat-denaturation in the SCSA as compared to normozoospermic samples (Evenson et al., 1980; Sailer et al., 1995). Thus, it appears that storage of sperm in the extra-epididymis produces nuclear changes similar to those already present in infertile sperm.

The mechanism by which sperm stored extra-epididymally develop an abnormal chromatin structure, as observed after vasectomy in this study, is unclear. Yanagida

et al. (1991) demonstrated that sperm lost the ability to decondense and form pronuclei after exposure to heat at 60-120°C for between 20 and 120 minutes, and they concluded that mature, mammalian sperm were moderately resistant to heat treatment. Addition of the reducing agent, dithiothreitol, produced an increased sensitivity of the sperm nuclei to thermal treatment, suggesting that the resistance of the cells to heat was related to the disulfide crosslinking of sperm protamines. After heating sperm at 100°C for 5 minutes, Evenson and colleagues (1980) demonstrated that mammalian sperm chromatin was susceptible to in situ heat denaturation. However, both of these studies exposed sperm to temperatures well in excess of those found at core body temperatures in vivo, albeit for short periods of time, which questions whether storage of sperm at 37°C would result in chromatin changes due to thermal exposure.

The chromatin changes observed in the study may be explained by a reduction of nuclear protamine disulphide bridges. Perreault et al. (1984, 1988) demonstrated that oocyte glutathione levels correlate positively with sperm nuclear decondensation, indicating that higher levels of glutathione result in a greater reduction of protamine disulphide bonds. In addition, glutathione reductase has been positively correlated with male pronucleus formation (Lassalle and Testart, 1989). Both glutathione and glutathione reductase are present in the ejaculate of vasectomized men, demonstrating extra-epididymal sources of the substances. The potential for proteolytic degradation of sperm nuclear proteins extra-epididymally should also be considered. Protease treatment of sperm in vitro produces nuclear decondensation (Gall and Ohsumi, 1976). The rate at which reduction of protamine disulphide bridges and proteolytic

degradation occurs would be expected to be greater at the higher temperatures extra-epididymal sperm are exposed to, as compared to testicular temperatures. Indeed, Reyes et al. (1996) established that decondensation with heparin and glutathione was significantly faster at higher temperatures. As reported by Jouannet and David (1969), semen obtained post-vasectomy in this study did not contain higher numbers of white blood cells compared to normozoospermic seminal fluid; therefore, surgical trauma does not seem a likely candidate to explain the adverse effects observed in the cells chromatin.

Whether the changes observed in this study are relevant to the normal ageing process of sperm in non-vasectomized men cannot be discounted. As much as a third of sperm present in the normal ejaculate emanate from sites distal to the epididymis (Freund and Davis, 1969; Jouannet and David, 1978). The changes observed in the current study took place within two weeks of vasectomy; therefore, the accumulation of sperm in the vas deferans through previous ejaculations or spontaneous leakage from the epididymis and subsequent storage for a similar period of time might be expected to produce comparable chromatin changes.

Previous studies have also reported lower total antioxidant activity in the semen of infertile men compared to that from normozoospermic donors (Lewis et al., 1995; Smith et al., 1996), in addition to higher levels of lipid peroxidation (Sanocka et al., 1996) and ROS (Alkan et al., 1997). The abundance of antioxidants and oxidative stress in the epididymis, therefore, may be an important determinant in male fertility.

6. Analytical assessment of seminal plasma: associations with smoking and antioxidants

6.1 Introduction

Studies on semen quality collated from several reports indicate a reduction in sperm count and semen volume over the past fifty years (Carlsen et al., 1992). Various environmental factors have been postulated to contribute to such a decrease in male fertility, including exposure to oestrogen mimicking compounds, pharmaceutical drugs and social factors, such as cigarette smoking and alcohol consumption. The measurement of compounds in semen that may be detrimental to sperm, causing reduced fertility or genetic damage for example, is therefore, of importance.

6.1.1 *Rate of Drug or Chemical Entry into Seminal Fluid*

Generally, the passage of a compound from blood or plasma into semen occurs by passive diffusion, the rate at which is dependent on its physicochemical characteristics, such as molecular weight, lipid solubility, protein binding and dissociation constant (Pichini et al., 1996). The blood-testes barrier also acts as a specialised anatomic barrier to molecules. The rate of excretion of a compound into semen is determined predominantly by its dissociation constant, which in turn determines the concentration of undissociated compound, and the lipid solubility of the undissociated molecule. The ratio of a compound present in blood plasma compared to that of a biological matrix, such as semen, can be predicted using the Henderson-Hasselbach equation:

$$\frac{C_M}{C_P} = \frac{1 + 10(pH_M - pK_a)}{1 + 10(pH_P - pK_a)} \quad \text{(for a weak acid)}$$

$$\frac{C_M}{C_P} = \frac{1 + 10(pK_a - pH_M)}{1 + 10(pK_a - pH_P)} \quad \text{(for a weak base)}$$

where:

C_M and C_P represent the total concentration of compound in biological matrix and plasma, respectively;

pK_a is the negative logarithm of the acid dissociation constant for each drug;

pH_M and pH_P are the negative logarithm of the hydrogen ion concentration in matrix and in plasma, respectively.

In accordance with the Henderson-Hasselbach equations, unbound molecules that are weak acids will concentrate in matrices whose pH is higher than that of plasma. The converse situation is true for weak bases. The matrix : plasma concentration will be near equal for compounds that are neither basic nor acidic.

6.1.2 *Methods for the Extraction of Compounds from Seminal Fluid*

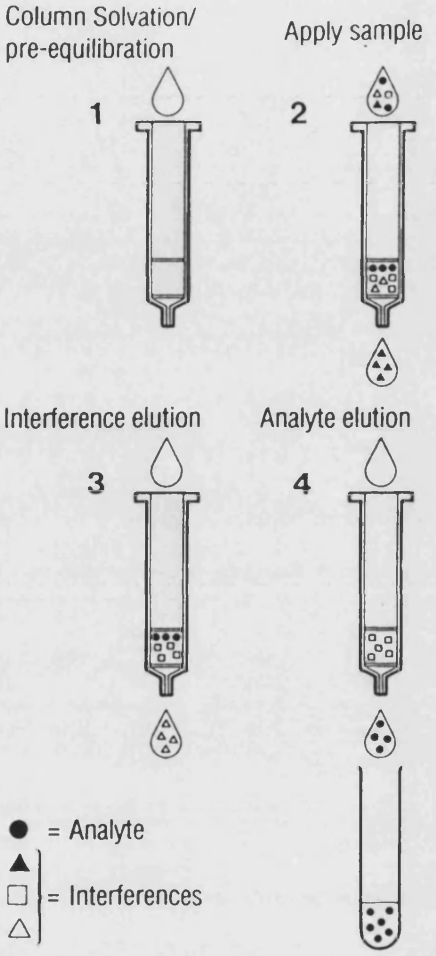
Several methods exist for the extraction of compounds from many different matrices. One of the most common and simpler methods is liquid-liquid extraction. The partitioning of compounds into different solvents, allows the experimenter to selectively use a liquid that the compound(s) of interest will partition into, leaving in

the remaining solvent as many interfering compounds as possible. Liquid-liquid extraction is relatively cheap, simple and effective. However, several drawbacks exist in the use of liquid-liquid extraction, such as an environmentally sound and economically viable method for the disposal of potentially large quantities of discarded solvents. The extracts produced are usually of a high volume and low concentration, often requiring long delays in analysis of the extract due to the necessity of concentrating the extraction solvent, which may result in the loss of volatile or unstable analytes. In addition, liquid extractions require extensive handling of samples which increases the potential for experimental error.

Solid phase extraction (SPE) is a technique based on the solubility and functional group interactions of an analyte, sorbent and solvent. In SPE, these interactions are utilised in order to isolate an analyte of interest on the sorbent and remove impurities and/or interferences with a suitable solvent. The analyte is then eluted from the sorbent using a small volume of solvent. When SPE columns are used optimally, a purified, concentrated solution of the analyte, free of compounds that would interfere with the qualitation and quantitation of the analyte, is produced.

An SPE procedure typically consists of four sequential steps, as depicted in fig. 6.1.

Fig. 6.1 Typical steps that are to be carried out during solid phase extraction (SPE) (source: International Sorbent Technology, 1993).

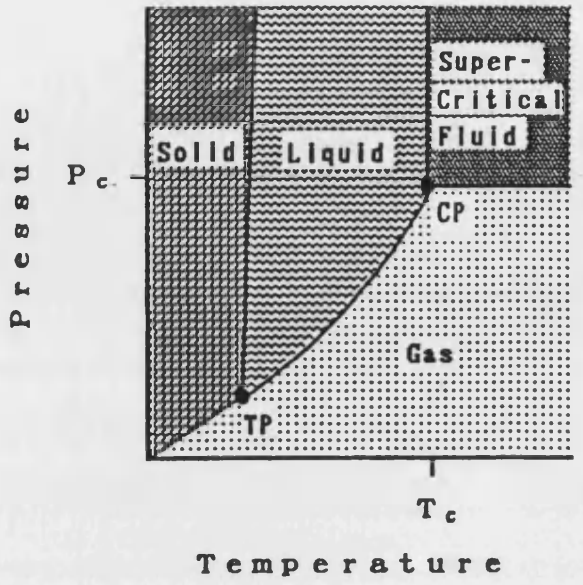


1. SPE column conditioning
2. Application of the sample to the column
3. Washing of the column to remove interferences
4. Elution of the analyte from the column

The majority of SPE procedures are now performed using disposable polypropylene columns, pre-packed with non-polar, polar or ion exchange sorbents. Common non-polar sorbents are octadecyl (C18), octyl (C8) and phenyl groups. The most commonly used polar sorbents are based on silica. Ion exchange sorbents can be either anion exchange, such as those containing amino groups, or cation exchange, for instance, those possessing carboxylic acid groups. The choice of sorbent is dependent on the analyte of interest and the matrix from which it is being removed. For example, a non-polar analyte can be extracted from a polar solution using a non-polar sorbent. After applying the solution to the SPE cartridge, the column can be washed with a polar solvent thus removing polar interferences from the sorbent, whilst retaining the analyte. The analyte can then be eluted using a small volume of a non-polar solvent.

Supercritical fluid extraction (SFE) technology allows the efficient, rapid extraction of many compounds from various matrices. A supercritical fluid is a substance above its critical temperature and pressure. Fig. 6.1 displays the pressure-temperature phase of a pure substance, in which the regions correspond to solid, liquid and gas. If we move from the triple point (TP) to the supercritical point (CP), both pressure and temperature increase; the liquid becomes less dense due to thermal expansion and the gas becomes more dense as the pressure increases. At the critical point, CP, the densities of the two phases become identical and the distinction between the gas and liquid disappear. Above its critical temperature, the substance does not undergo a transition from liquid to gas or vice-versa. The substance in this region is said to be a supercritical fluid (Saito and Yamuchi, 1990).

Fig. 6.2. Temperature – pressure phase diagram of a pure substance (source: Saito and Yamuchi, 1990).



In order to be used in SFE, a fluid must possess solvating power. Table 6.1 shows the properties of a gas, a liquid and a supercritical fluid. The diffusivity of a supercritical fluid is greater than that of a liquid by several hundred-fold. This results in faster mass transport compared to a liquid by the same factor. Viscosity is also a hundred times lower than a liquid, although the density is similar. The kinetic viscosity is defined as the viscosity divided by the density; thus the kinetic viscosity of a supercritical fluid is very low compared with that of a liquid. As a consequence, the Reynolds number, defined as the inertial force divided by the viscous force, is 30 to 100 times higher for a supercritical fluid than that of a liquid for a given flow velocity. This increases turbulent flow in tubing and reduces the laminar flow

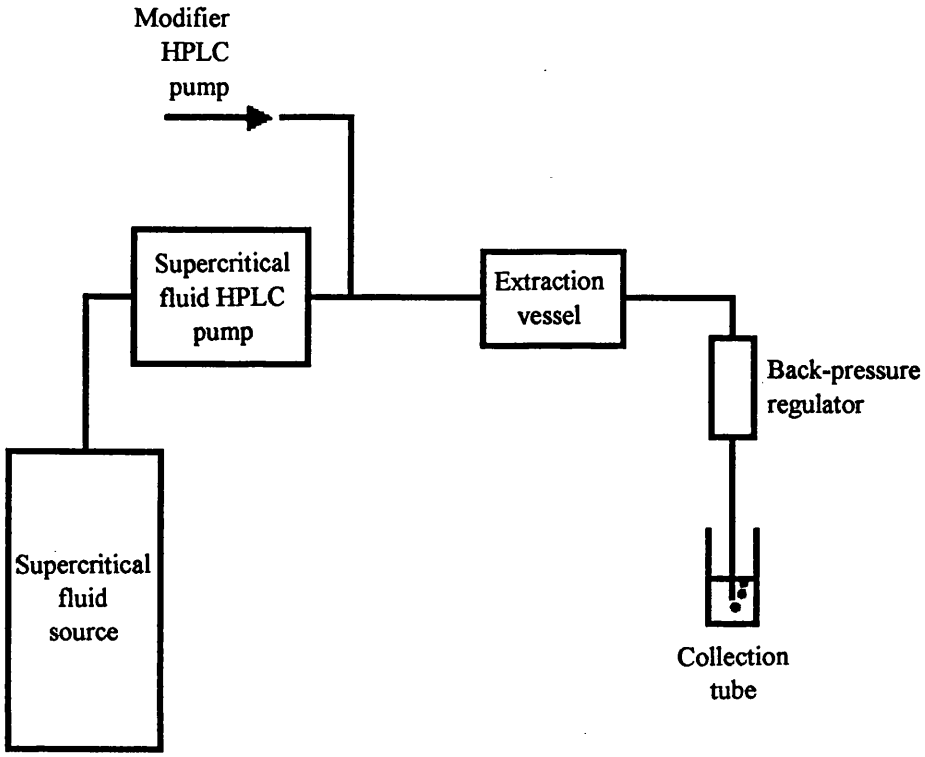
dispersion. Overall, the mass transfer in an extraction employing a supercritical fluid solvent compared with a liquid solvent becomes much faster, offering a far higher throughput.

Table 6.1. Properties of a gas, a liquid and a supercritical fluid (source: Saito and Yamuchi, 1990).

Property	Gas	Liquid	Supercritical Fluid
Density ($g.cm^{-3}$)	10^{-3}	1	0.3
Diffusivity ($cm^2.s^{-1}$)	10^{-1}	5×10^{-6}	10^{-3}
Viscosity ($g.cm^{-1}.s^{-1}$)	10^{-4}	10^{-2}	10^{-4}

A typical SFE system is comprised of a high-pressure pump(s), an extraction vessel that is contained within an oven and a back-pressure regulator, as shown in fig. 6.3.

Fig. 6.3. A typical set-up for a SFE system. Note that the back-pressure regulator requires heating in order to prevent plugging by frozen CO_2.



The back-pressure regulator ensures that the pressure of the entire system is maintained above the critical pressure of the fluid. Likewise, the extraction vessel is heated to ensure that the temperature of the solvent is above that of the critical temperature of the fluid.

The most commonly used substance as a supercritical fluid is carbon dioxide (Saito and Yamuchi, 1990). Carbon dioxide is relatively non-polar and can, therefore, be used for the extraction of many organic solutes. It is also relatively cheap and non-toxic. Modifiers such as alcohols can be added to the supercritical fluid for the extraction of more polar compounds.

6.2 Methods

6.2.1 *Semen Samples*

Semen samples were obtained from patients being screened for fertility at the Royal United Hospital, Bath, UK. All samples were collected by masturbation after 3 days of abstinence. Following liquefaction at room temperature for 1 h, semen analysis was performed by staff of the Royal United Hospital (only those classified as normozoospermic as defined by the World Health Organization (WHO 1992) were included in the study; see section 2.2.1). All samples were stored at -20°C prior to use. The smoking status of each donor was recorded and the samples used in the study characterised as either non-smokers or smokers of more than 5 cigarettes per day. This study had the approval of the Bath District Ethics Committee.

All materials were obtained from Sigma-Aldrich unless otherwise noted (Sigma-Aldrich Chemicals, Poole, Dorset, UK), with the exception of solvents, which were obtained from BDH (BDH Laboratory Supplies, Poole, Dorset, UK).

6.2.2 *Statistical Methods*

The data obtained using the above assays were not normally distributed (Anderson-Darling test for normality). Therefore, the non-parametric Mann-Whitney *U* test was used to examine differences between samples from smoking and non-smoking donors, where a probability value of $P<0.05$ was considered significant. The statistical analyses were carried out using Minitab 11.12 (Minitab Inc., Pennsylvania, USA).

Section A: Detection of Compounds in Seminal Fluid using Supercritical Fluid Extraction Technology

A.6.1 Introduction

Several studies have indicated an adverse effect of smoking on the male reproductive system, including spermatozoal DNA damage (Fraga et al., 1996; Shen et al., 1997), increased levels of sperm aneuploidy (Wyrobek et al., 1995), and a reduction in sperm concentration and motility in the ejaculate (Pacifici et al., 1993; Sofikitis et al., 1995; Vine et al., 1994; Vine et al., 1996). Additionally, paternal cigarette smoking has been associated with certain childhood cancers and birth defects (Zhang et al., 1992; Sorahan et al., 1995; 1997a; 1997b). Furthermore, partners of male smokers are reportably at greater risk of developing cervical cancer (Bosch et al., 1996).

The purpose of the current study was to extract large volumes of seminal fluid so that identification of potentially toxic compounds could be performed from semen obtained from donors who smoked, and those that did not. Semen would be extracted using SFE, and the extracts would then be subjected to liquid-liquid extraction so as to aid in the resolution and identification of analytes, prior to GC-MS analysis. We report that several hydrocarbons were identified in seminal fluid. In addition, numerous cigarette smoke components and biological compounds were also positively identified.

A.6.2 Methods

A.6.2.1 *Extraction of Compounds in Seminal Fluid using SFE*

The extraction of organic compounds from seminal fluid was accomplished using a Jasco SFE system (Jasco UK, Great Dunmow, UK) using CO_2 as supercritical fluid at a flow rate of 3 ml/min and methanol as modifier at a flow rate of 0.3 ml/min. The entire system was maintained at a pressure of 200 kg/cm^2. An oven temperature of 50°C was implemented.

Seminal fluid was thawed at room temperature and samples obtained from smokers and non-smokers each amalgamated separately to a volume of 30 ml. The plasma was deproteinated by admixing with 60 ml acetonitrile and the supernatant collected after centrifugation at 2000 x g for 10 min. The supernatant was mixed with 20 g α-cellulose and allowed to dry in a fume cupboard for 12 h at room temperature.

After drying, the packing material was placed into a 40 ml extraction vessel. The extraction vessel was topped up with α-cellulose and connected inside the SFE oven. The extraction vessel was then left inside the oven for 20 min at 50°C to allow temperature equilibration.

Once equilibrated, the CO_2 and modifier pumps were turned on and allowed to pressurise, whilst being isolated from the oven. Once pressurised, the extraction fluid was allowed to enter the oven and flow through the extraction vessel. The eluate was collected for 60 min in 1 ml acetone, that was stored on ice and protected from light.

Once the extraction was completed, the acetone containing the extract was shaken with an equal volume of *n*-hexane. The mixture was centrifuged at 1000 x g for 10 min and the *n*-hexane layer removed. This extraction was repeated and the *n*-hexane

extracts combined. The acetone extract was then further extracted with two equal volumes of chloroform, as performed using *n*-hexane.

The acetone, *n*-hexane and chloroform extracts were dried using a rotary evaporator at room temperature, and the residue dissolved in 1 ml heptane. The heptane was then concentrated to a volume of 50 µl, prior to chromatographic analysis.

A.6.2.2 *Chromatographic Analysis*

The SFE extracts were analysed using gas chromatography combined with a mass spectrometer (GC-MS). GC-MS was carried out using a Hewlett Packard 5970 gas chromatographer (Hewlett Packard, Pennsylvania, USA) fitted with a 40 m BPX5 column (SGE Chromatography, Milton Keynes, UK), and Hewlett Packard 5890 mass spectrometer (Hewlett Packard, Pennsylvania, USA). Helium was used as the carrier gas, at a flow of 100 ml/min. A cold on-column splitless injector was used, whilst the detector and interface were held at 280°C. A 1.5 µl injection volume was used for each analysis. At the point of injection, the oven temperature was 60°C. Subsequently, the oven remained at 60°C for 2 min, before rising to 250°C at a rate of 3°C per min. Data were acquired using Standard ChemStation software (Hewlett Packard, Pennsylvania, USA, 1996).

A.6.3 Results

A.6.3.1 *Extraction of Compounds in Seminal Fluid using SFE*

The initial SFE extract collected in acetone, was extracted sequentially with *n*-hexane followed by chloroform. The use of *n*-hexane allowed the extraction of non-polar hydrocarbons from the initial SFE extract. Chloroform allowed the removal of more polar compounds from the initial SFE extract. The use of liquid-liquid extraction enabled more precise determination of individual components in the chromatogram, due to the removal of many potential interfering compounds with each subsequent solvent extraction.

Each extract was injected into a GC-MS, which allowed a more precise identification of compounds through comparing their individual mass spectra with those stored in the ChemStation software database.

The *n*-hexane extracts typically contained many long-chain hydrocarbons, identified according to their mass spectra (see fig. A.6.1-A.6.2). The aromatic hydrocarbon, 1,2 benzenedicarboxylic acid, was also present in the *n*-hexane extract of both smokers and non-smokers semen.

Fig. A.6.1. Typical chromatogram using gas chromatography with mass spectrometry detection of a 30 ml seminal fluid extract, obtained from men who were cigarette smokers. The extract was collected in acetone and analysed as described under materials and methods, section A.6.2, using supercritical fluid extraction (SFE). The acetone fraction was then subsequently subjected to *n*-hexane and chloroform liquid-liquid extraction. This chromatogram represents the components contained in the *n*-hexane fraction. Compounds that were identifiable on the chromatogram according to their mass spectra are labelled.

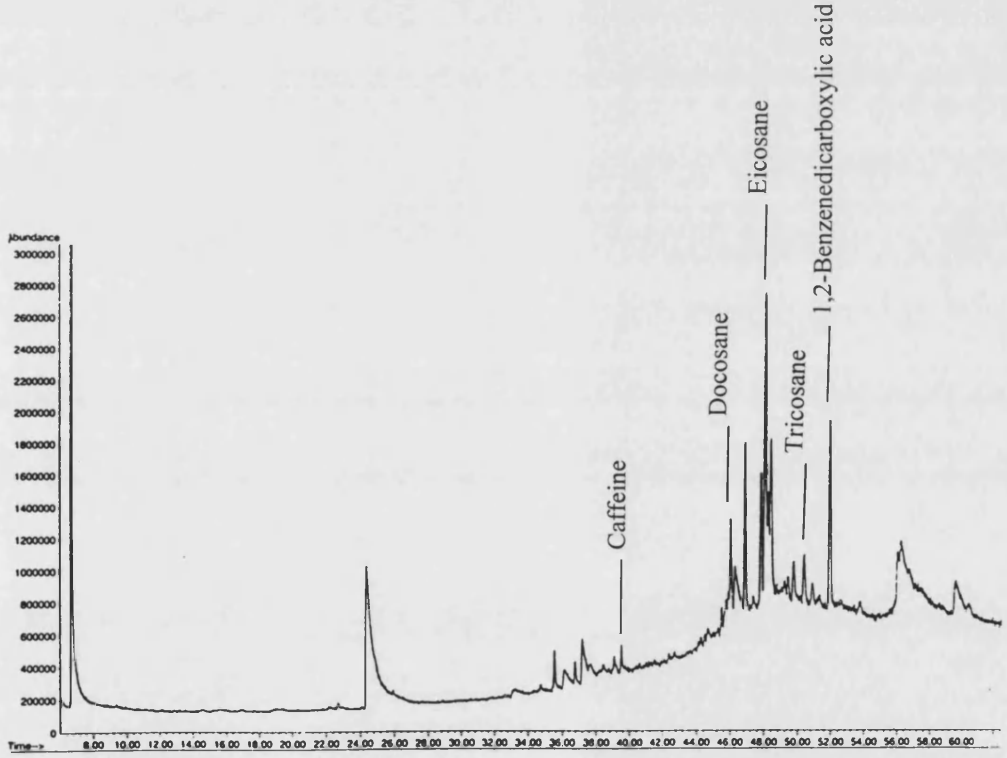


Fig. A.6.2. Several mass spectra obtained from a chromatogram using gas chromatography with mass spectrometry detection of a 30 ml seminal fluid extract. The extract was processed as described for fig. A.6.1. The mass spectra were produced from peaks contained in the *n*-hexane fraction.

Fig. A.6.2a. Mass spectra of eicosane, that was present in the semen of both smokers and non-smokers, produced after SFE of 30 ml of semen. The uppermost spectra is that obtained from the samples chromatogram after GC-MS analysis, whilst the lower panel contains the spectra for eicosane retrieved from the ChemStation software database.

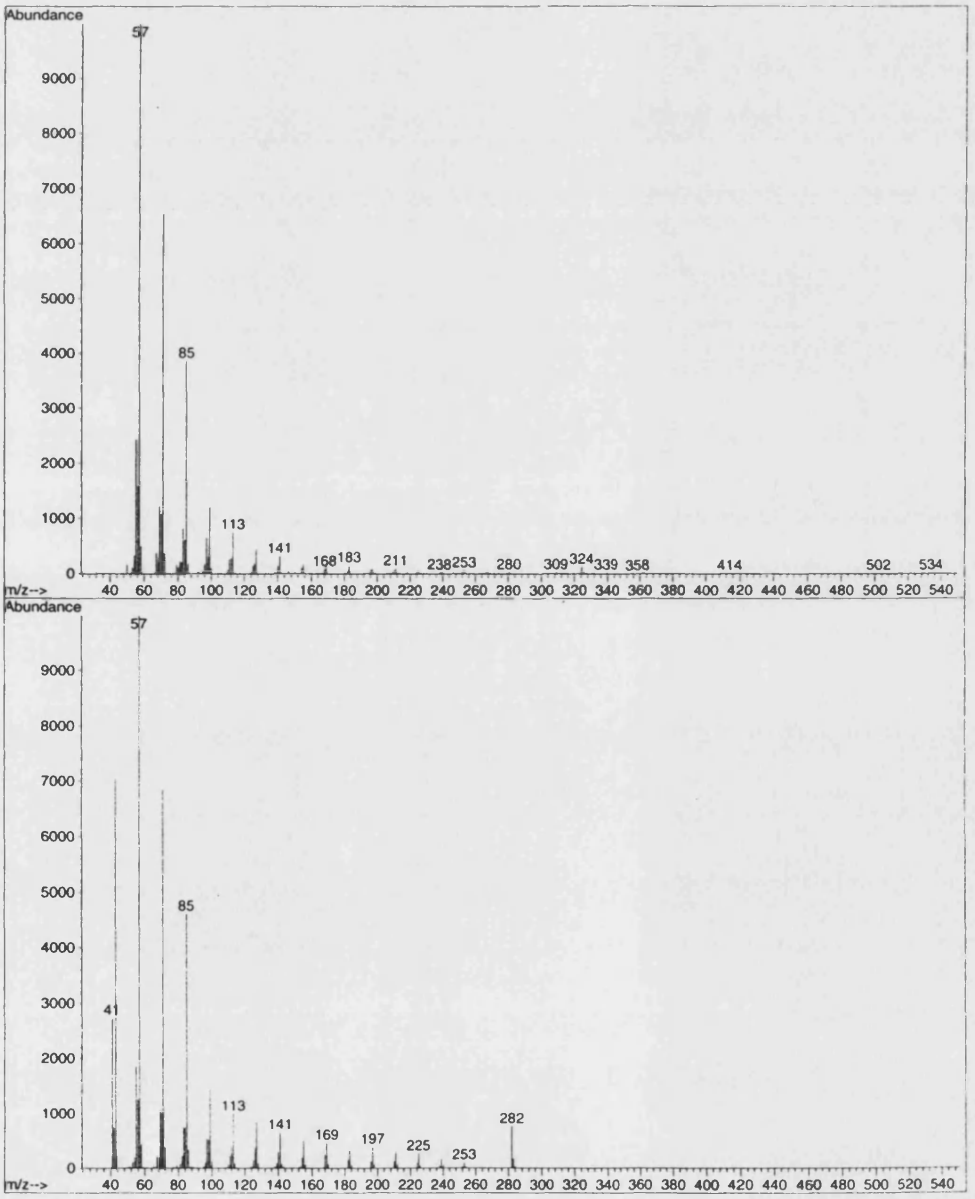


Fig. A.6.2b. Mass spectra of octadecanoic acid, that was present in the semen of both smokers and non-smokers, produced after SFE of 30 ml of semen. The uppermost spectra is that obtained from the samples chromatogram after GC-MS analysis, whilst the lower panel contains the spectra for octadecanoic acid retrieved from the ChemStation software database.

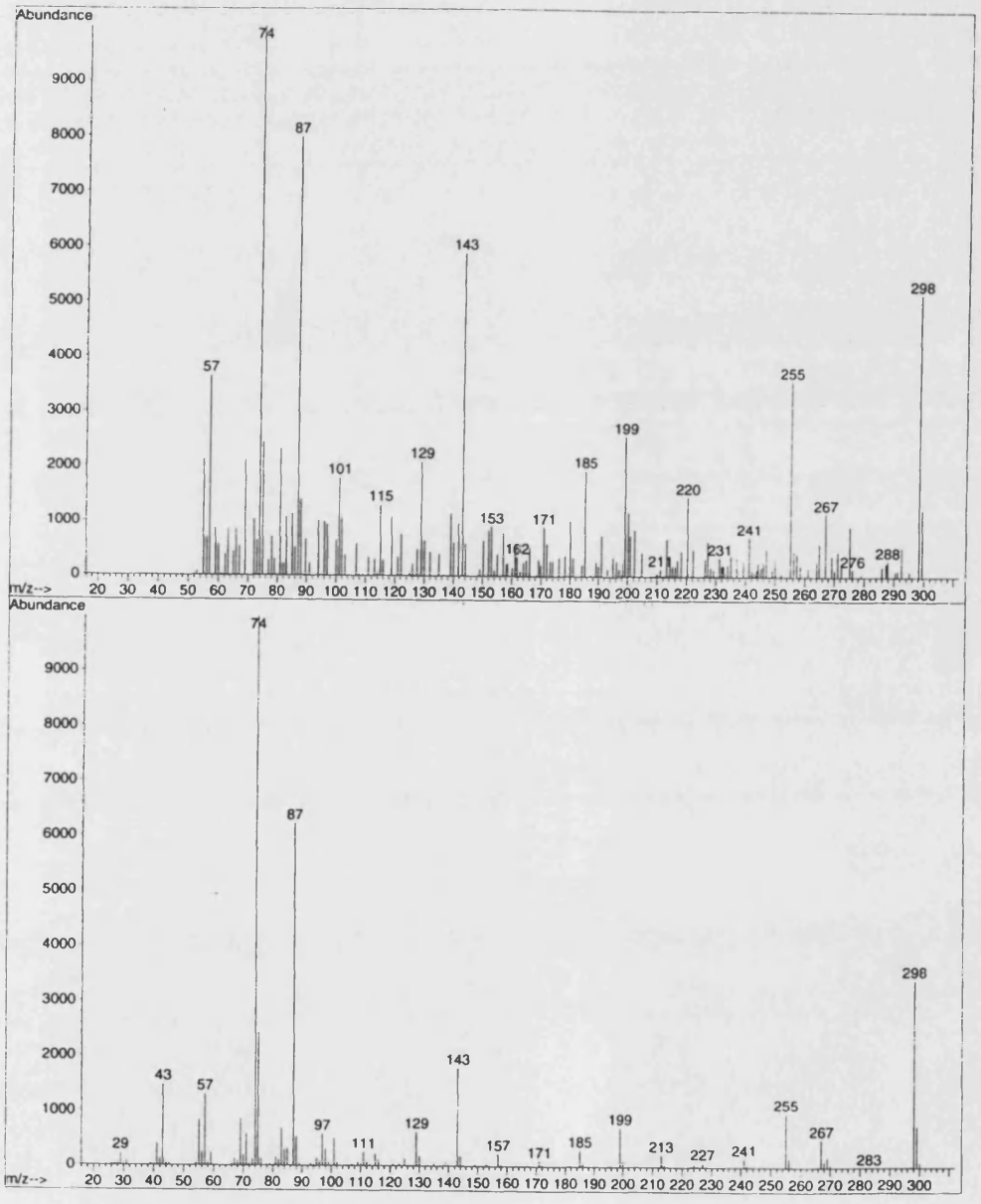
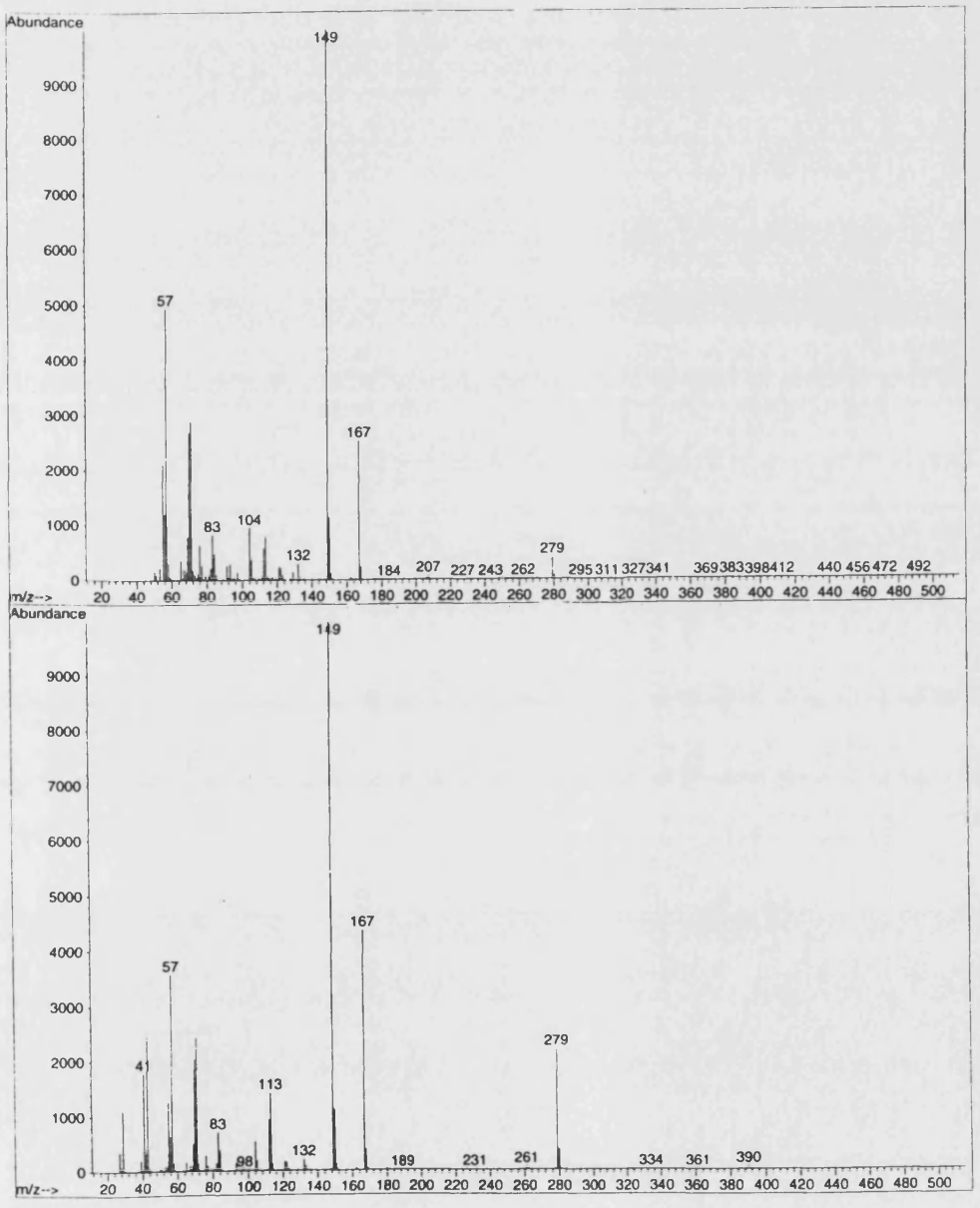


Fig. A.6.2c. Mass spectra of the aromatic hydrocarbon, 1,2 benzenedicarboxylic acid, that was present in the semen of both smokers and non-smokers, produced after SFE of 30 ml of semen. The uppermost spectra is that obtained from the samples chromatogram after GC-MS analysis, whilst the lower panel contains the spectra for 1,2 benzenedicarboxylic acid retrieved from the ChemStation software database.



The chloroform extracts contained detectable quantities of caffeine, as well as cholesterol, in both smokers and non-smokers semen (see fig. A.6.3-A.6.5). Nicotine and cotinine were also present in the seminal fluid obtained from smokers.

Fig. A.6.3. Typical chromatogram using gas chromatography with mass spectrometry detection of a 30 ml seminal fluid extract, obtained from men who were cigarette smokers. The extract was collected in acetone and analysed as described under materials and methods, section A.6.2, using supercritical fluid extraction (SFE). The acetone fraction was then subsequently subjected to *n*-hexane and chloroform liquid-liquid extraction. This chromatogram represents the components contained in the chloroform fraction. Compounds that were identifiable on the chromatogram according to their mass spectra are labelled.

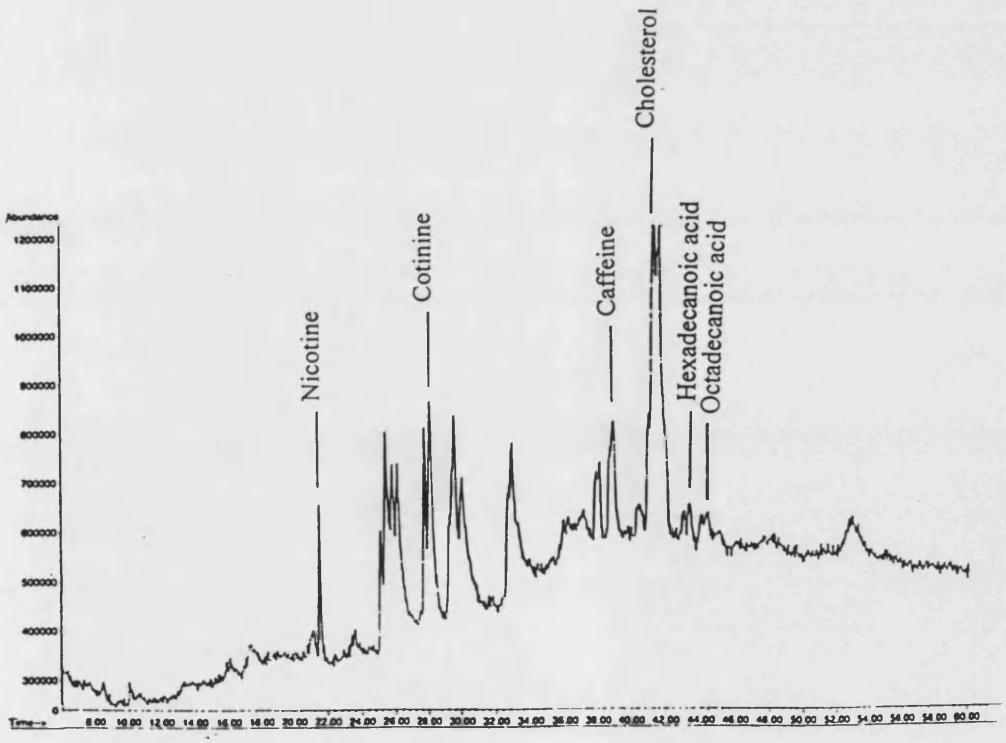


Fig. A.6.4. Typical chromatogram using gas chromatography with mass spectrometry detection of a 30 ml seminal fluid extract, obtained from men who were not cigarette smokers. The extract was processed as described as for fig. A.6.3. Compounds that were identifiable on the chromatogram according to their mass spectra are labelled.

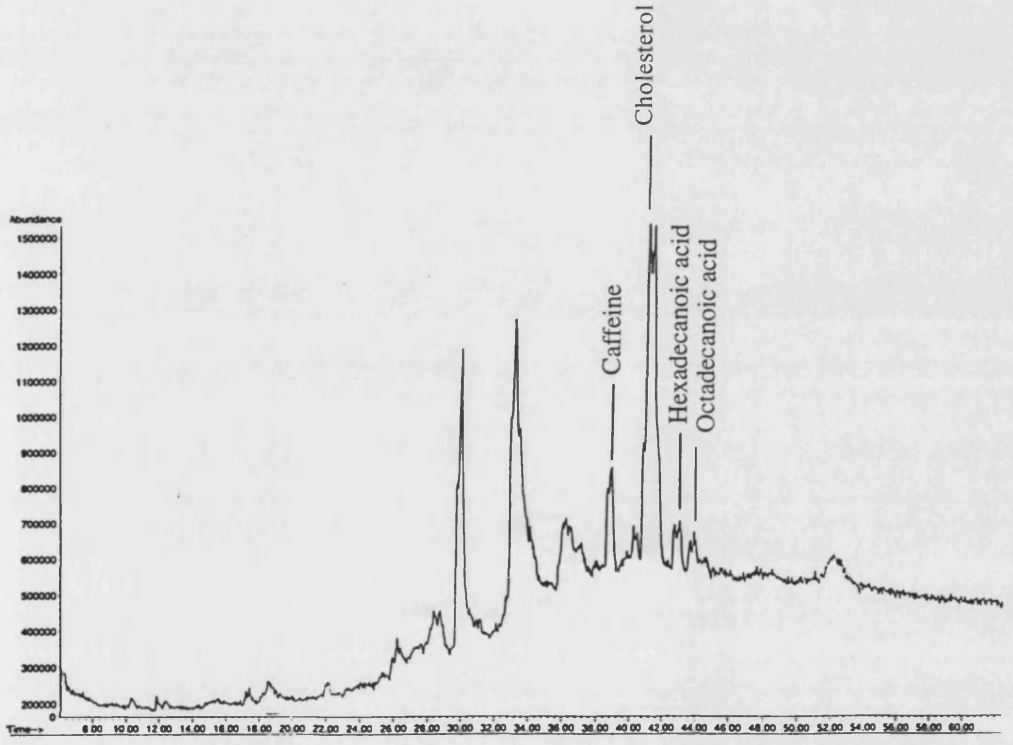


Fig. A.6.5. Several mass spectra obtained from a chromatogram using gas chromatography with mass spectrometry detection of a 30 ml seminal fluid extract, obtained from men who were cigarette smokers. The extract was processed as described as for fig. A.6.3. The mass spectra were produced from peaks contained in the chloroform fraction.

Fig. A.6.5a. Mass spectra of nicotine, produced after SFE of 30 ml of semen obtained from men who smoked. The uppermost spectra is that obtained from the samples chromatogram after GC-MS analysis, whilst the lower panel contains the spectra for nicotine retrieved from the ChemStation software database.

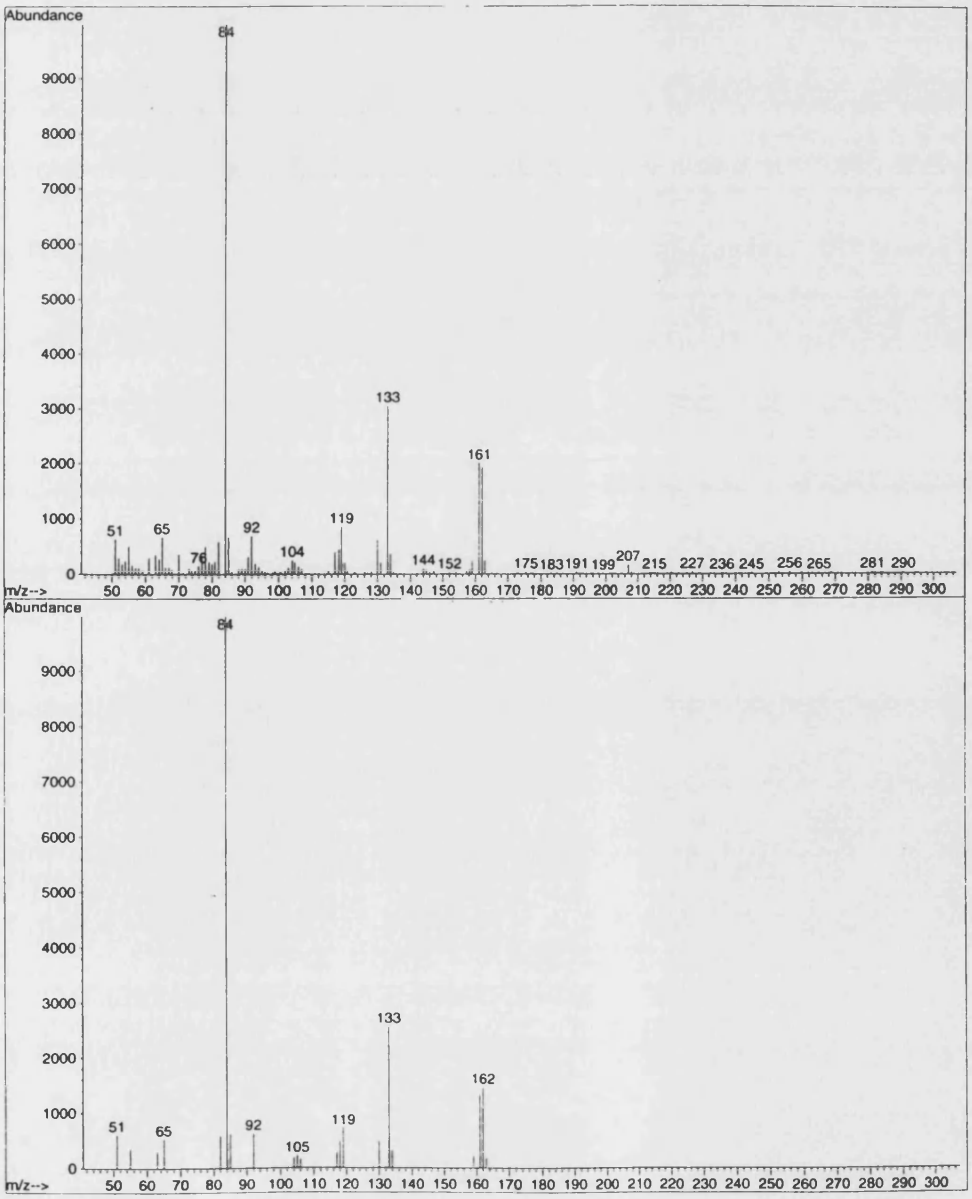


Fig. A.6.5b. Mass spectra of cotinine, produced after SFE of 30 ml of semen obtained from men who smoked. The uppermost spectra is that obtained from the samples chromatogram after GC-MS analysis, whilst the lower panel contains the spectra for cotinine retrieved from the ChemStation software database.

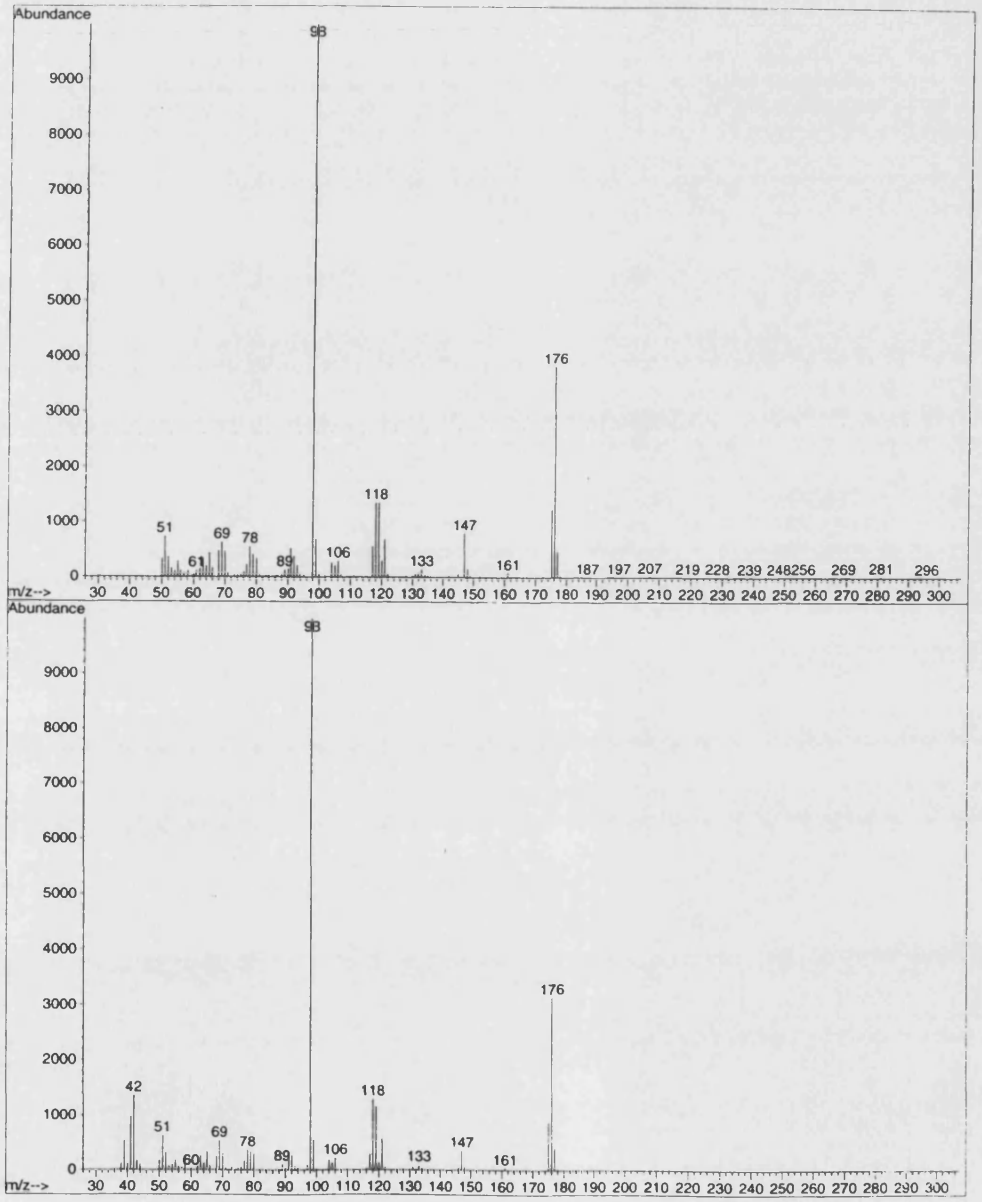


Fig. A.6.5c. Mass spectra of caffeine, produced after SFE of 30 ml of semen obtained from men who smoked. The uppermost spectra is that obtained from the samples chromatogram after GC-MS analysis, whilst the lower panel contains the spectra for caffeine retrieved from the ChemStation software database.

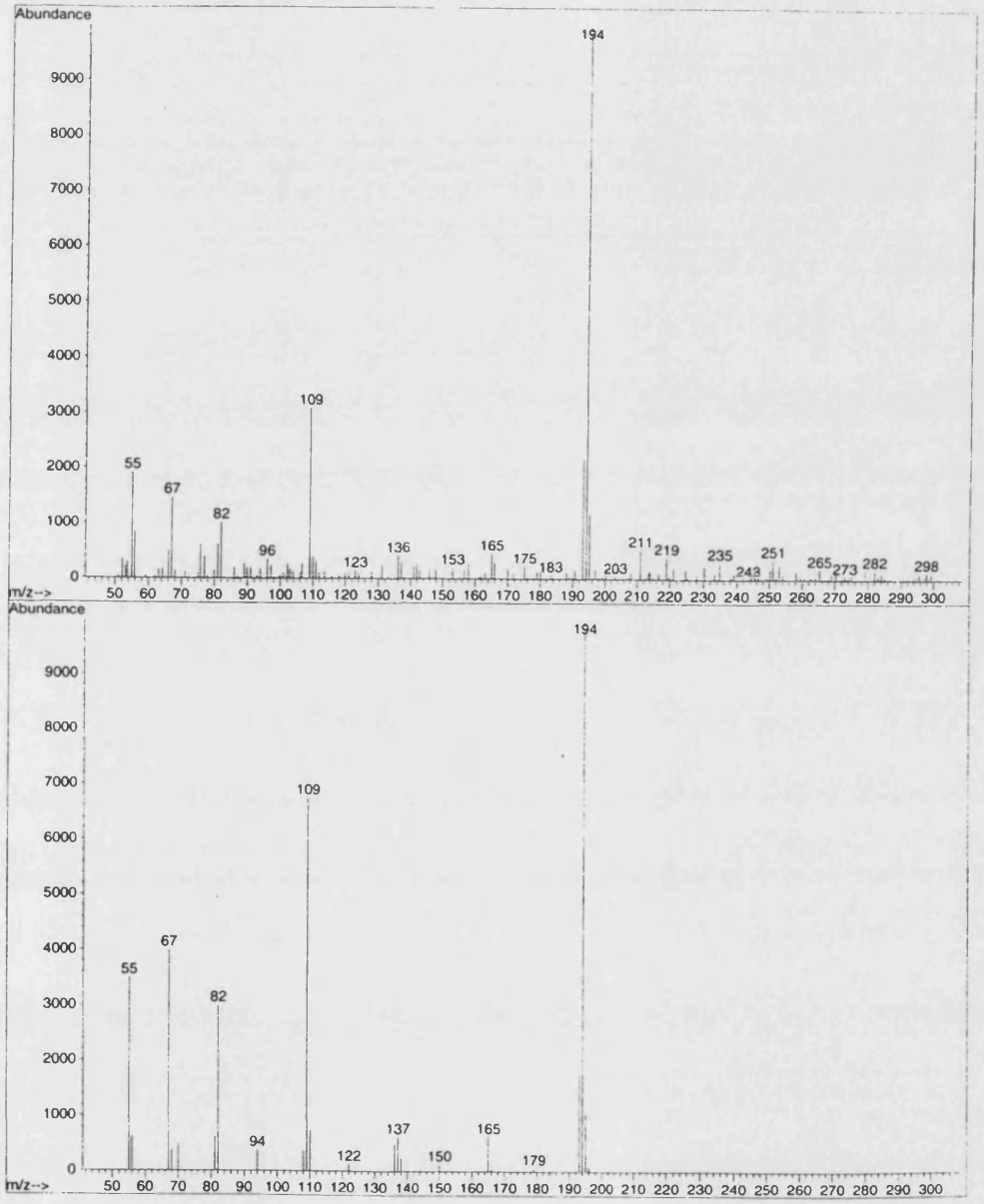
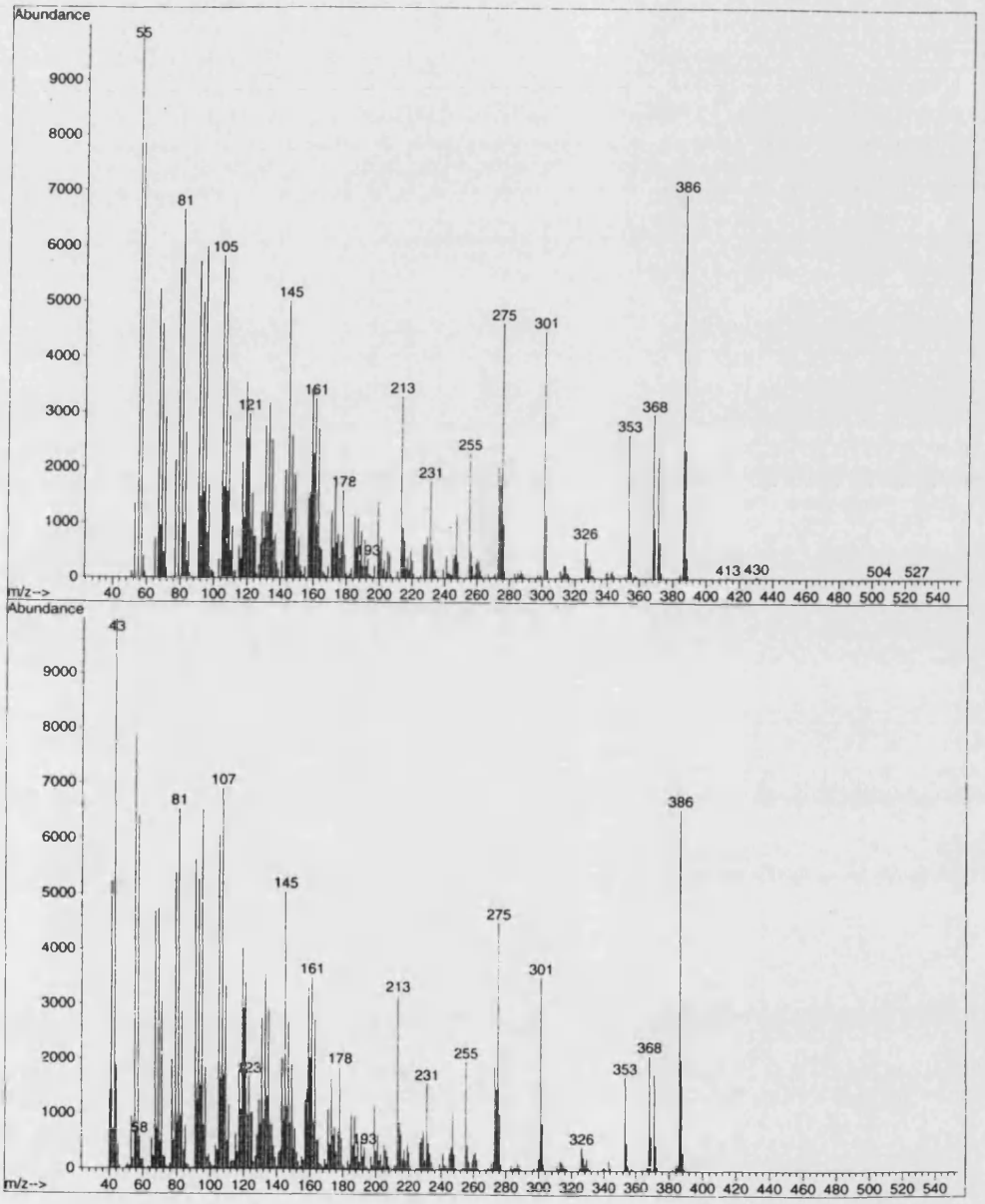


Fig. A.6.5d. Mass spectra of cholesterol, produced after SFE of 30 ml of semen obtained from men who smoked. The uppermost spectra is that obtained from the samples chromatogram after GC-MS analysis, whilst the lower panel contains the spectra for cholesterol retrieved from the ChemStation software database.



The components that remained in the acetone following *n*-hexane and chloroform extraction, did not contain any identifiable compounds. This was thought to be due to the polar nature of the extract. In order to improve the chromatography of the extract, the sample was derivatised with bis(trimethylsilyl)trifluoro-acetamide (BSTFA). Derivatisation was performed by first drying the sample under a stream of nitrogen, and resuspending the extract in 200 µl 50 % BSTFA in ethyl acetate. The solution was then heated for 2 hours at 100°C. The sample was again dried under a stream of nitrogen and resuspended in 50 µl heptane, prior to GC-MS analysis. This did not, however, improve the GC-MS analysis, and therefore, the results cannot be discussed.

A.6.4 Discussion

This study utilised SFE technology and GC-MS in order to assess whether semen contains compounds that are capable of damaging the male reproductive system and/or producing toxic responses in the female reproductive tract upon exposure to semen. The results demonstrate the presence of caffeine in semen, as well as nicotine and cotinine in the semen of men who smoke. Several biological components were also positively identified in both smokers and non-smokers seminal fluid, including cholesterol, and several hydrocarbons such as eicosane and tetracosane. No difference in the number or amount of hydrocarbons were observed between semen from smokers and semen from non-smokers. The aromatic hydrocarbon, 1,2 benzenedicarboxylic acid, was detected using GC-MS after the extraction of semen with SFE. Many aromatic compounds possess mutagenic and carcinogenic properties (Hoffman and Hecht, 1989; Brunnemann and Hoffman, 1992), and the toxic potential of this compound on the male and/or female reproductive tract cannot be discounted at this stage. However, 1,2 benzenedicarboxylic acid is used in the synthesis of plastics (Standard ChemStation software 1996). Therefore, the compound may be a component of the plastic containers that were used for the storage of semen, and not actually a normal resident of seminal fluid in vivo.

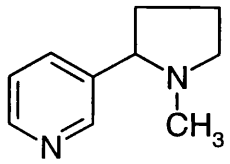
The confirmed presence of nicotine and cotinine cannot discount the possibility that nitrosamines may be present in semen, albeit at concentrations lower than those detectable using the current equipment. Testicular microsomes contain the hepatic biotransforming enzymes, the cytochrome P450 family (Parkinson 1996), thus activation of nicotine or cotinine to a mutagenic nitrosamine is plausible.

Section B: Measurement of organic Compounds in Seminal Fluid using Supercritical Fluid Extraction: Associations with Cigarette Smoking

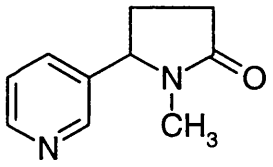
B.6.1 Introduction

The purpose of the current study was to develop a method based on SFE technology, that would enable an efficient and rapid extraction of organic compounds from seminal fluid. Specifically, we resolved to extract nicotine and one of its major metabolites in vivo, cotinine, as well as the commonly ingested food and drink component, caffeine, whose structures are shown in fig. B.6.1.

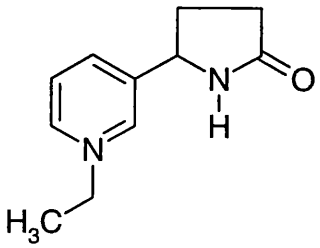
Fig. B.6.1. Structures of nicotine, cotinine and caffeine, as well as the internal standard used for the chromatographic analysis, N-ethylnorcotinine.



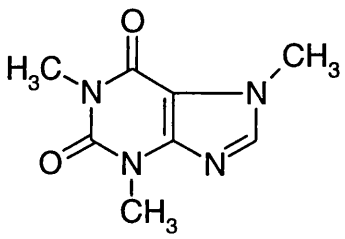
Nicotine



Cotinine



N-Ethylnorcotinine



Caffeine

We report a novel application of SFE for the extraction of several organic compounds from human seminal fluid, namely cotinine and caffeine. The method is rapid, reproducible and allows excellent recovery of analytes. However, nicotine was not adequately resolved in the chromatographic analysis to allow quantitation.

B.6.2 Methods

B.6.2.1 *Extraction of Organic Compounds in Seminal Fluid using SFE*

The extraction of organic compounds from seminal fluid was accomplished using SFE comprising CO_2 as supercritical fluid at a flow rate of 3 ml/min and methanol as modifier at a flow rate of 0.3 ml/min. The entire system was maintained at a pressure of 200 kg/cm^2, and an oven temperature of 50°C implemented.

To a 1 ml aliquot of seminal fluid was added 200 ng N-ethylnorcotinine in methanol to act as internal standard. The sample was deproteinated by admixing with an 2 ml acetonitrile and the supernatant collected after centrifugation at 2000 x g for 10 min. The supernatant was mixed with 1 g α-cellulose and allowed to dry in a fume cupboard for 2 h at room temperature.

After drying, the packing material was placed into a 10 ml extraction vessel. The extraction vessel was topped up with α-cellulose and connected inside the oven. The extraction vessel was then left inside the oven for 20 min at 50°C to allow temperature equilibration.

Once equilibrated, the CO_2 and modifier pumps were turned on and allowed to pressurise, whilst being isolated from the oven. Once pressurised, the extraction fluid was allowed to enter the oven and flow through the extraction vessel. The elute was collected for 30 min in 1 ml acetone, that was stored on ice and protected from light. The extract was dried under a gentle stream of nitrogen, and resuspended in 300 µl mobile phase prior to chromatographic analysis.

B.6.2.2 *Chromatographic Analysis*

The SFE extracts were analysed using HPLC with UV detection. The liquid chromatography system consisted of a Jasco PU-980 pump (Jasco UK, Great Dunmow, UK), a Hichrom 5 μm 150 x 4.6 mm C18 column equipped with a Hichrom guard column (5 μm, 10 x 4.6 mm; Hichrom Limited, Reading, UK), and a Jasco UV-975 detector set at 259 nm. The mobile phase consisted of 0.05 M sodium acetate containing water : acetonitrile (80 : 20 v/v), adjusted to pH 4.4 using acetic acid. The mobile phase was filtered through a 0.45 μm filter under vacuum and degassed using helium before use. A 50 μl injection volume was used for each extract. All separations were carried out at ambient room temperature using a flow rate of 1 ml/min.

B.6.3 Results

B.6.3.1 *Extraction of Organic Compounds in Seminal Fluid*

With the use of SFE technology, an excellent recovery of caffeine and cotinine from seminal plasma was attained. Cotinine was recovered at a level of 93.53 ± 2.29 %, and caffeine at 91.26 ± 2.84 %. Nicotine was poorly resolved by the chromatographic conditions employed and was not quantifiable.

Fig. B.6.2-B.6.3 displays the calibration curves produced following extraction of cotinine and caffeine from seminal fluid using SFE and HPLC analysis. The analysis was linear up to a concentration of 500 ng/ml cotinine or caffeine (r = 0.997).

Fig. B.6.2. Calibration curve for the analysis of cotinine from seminal fluid using SFE and HPLC analysis.

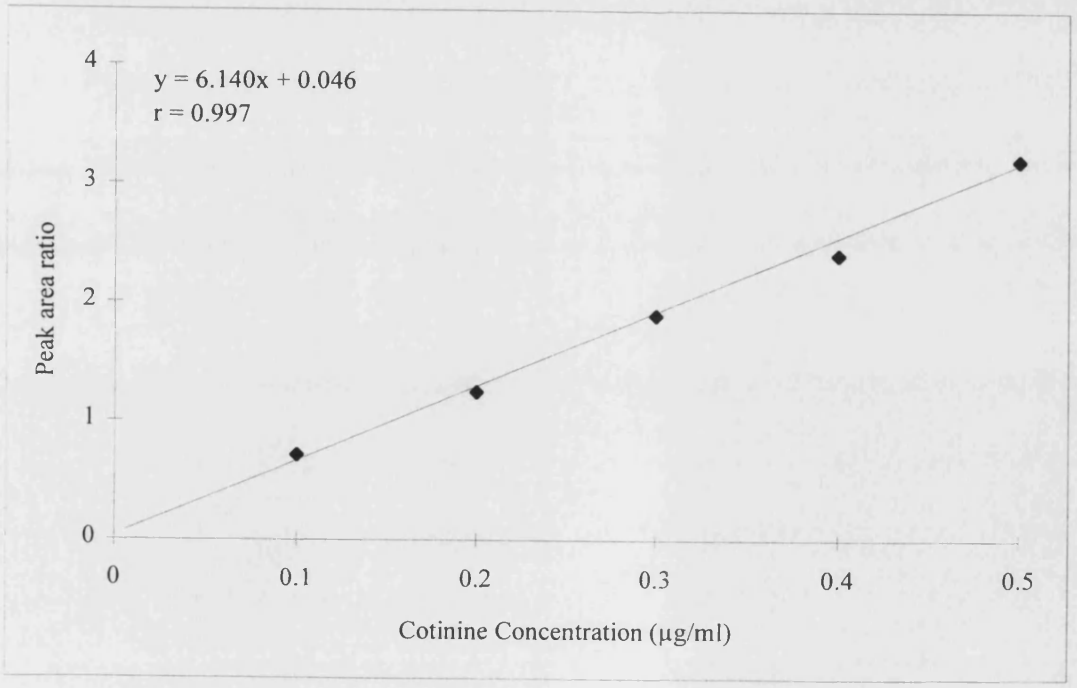
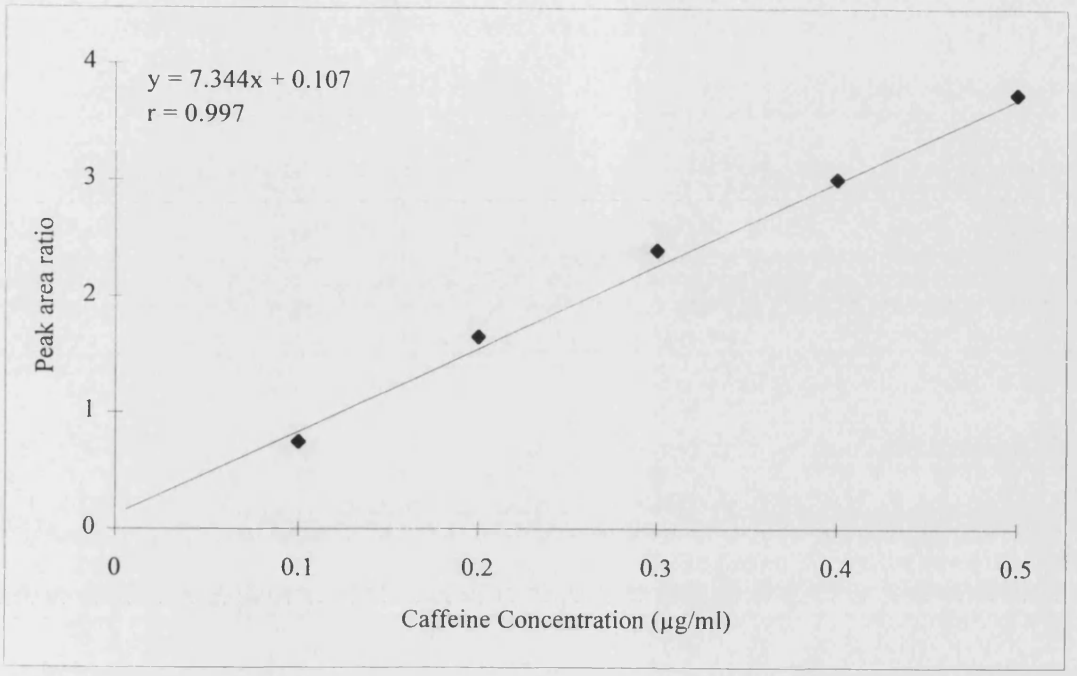
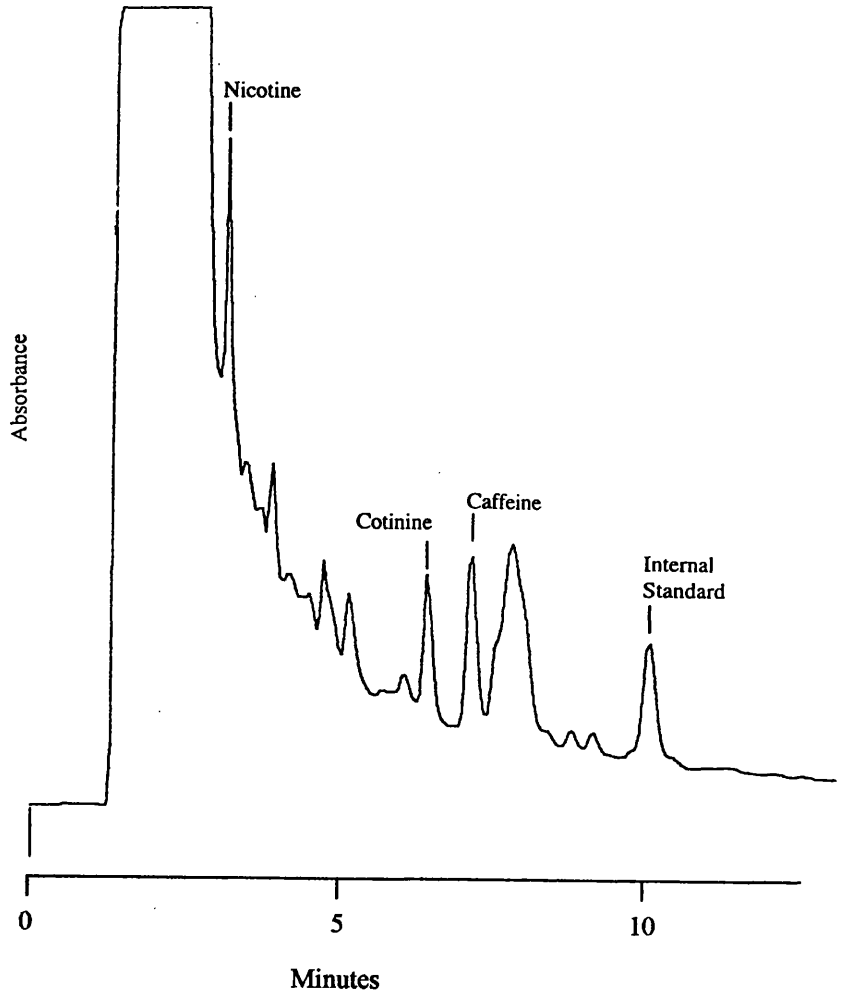


Fig. B.6.3. Calibration curve for the analysis of caffeine from seminal fluid using SFE and HPLC analysis.



Supercritical fluid extracts of seminal fluid did not enable adequate chromatographic resolution of nicotine (fig. B.6.4). Cotinine, however, was well resolved upon HPLC analysis. As cotinine is generally present at higher plasma concentrations than nicotine, is the major metabolite of nicotine produced in man, and provides a more accurate reflection of cigarette consumption due to a longer plasma half-life, further steps to improve the resolution of nicotine to enable its quantitation were not undertaken.

Fig. B.6.4. Typical chromatogram of seminal fluid obtained from a smoker, executed as described under materials and methods, section B.6.2, using supercritical fluid extraction and HPLC with UV detection, to allow the extraction and measurement of cotinine and caffeine. The mobile phase consisting of 0.05 M sodium acetate containing water : acetonitrile (80 : 20 v/v), pH 4.4, produced retention times of 7.28 min for cotinine, 9.14 min for caffeine and 10.18 min for the internal standard, N-ethylnorcotinine.



B.6.4 Discussion

SFE was applied to the extraction of compounds from seminal fluid, allowing the subsequent HPLC determination of cotinine and caffeine. This application of SFE, allowed an efficient assay to be developed for these compounds. As the supercritical fluid, CO_2, forms a gas once it has left the pressurised SFE system, leaving behind a concentrated elute, the extraction is relatively rapid to perform bearing in mind the negligible concentrating of sample that is required prior to analysis.

Previous studies have determined nicotine, cotinine and caffeine levels in seminal plasma, utilising diatomaceous earth filled columns to extract the compounds followed by HPLC determination (Pacifici et al., 1993). However, when the same extraction was applied in the current study followed by HPLC analysis, poor recovery was produced (less than 10 %). In addition, the extracts were insufficiently clean to resolve the peaks of interest. These problems were resolved in the current study by the use of supercritical fluid technology.

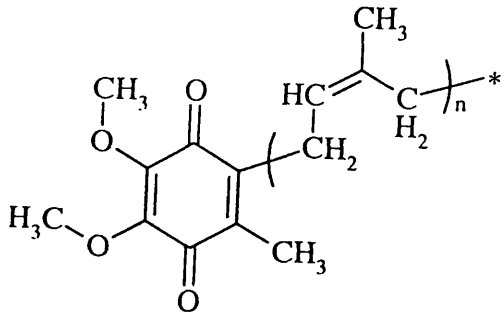
Cigarette smoking has been associated with poor semen quality and spermatozoal damage, as discussed previously. The consumption of caffeine has also been correlated with spermatozoal aneuploidy (Robbins et al., 1997). Methods that enable the effective determination of such compounds in seminal fluid is, therefore, of importance. Future studies should determine thoroughly using a large number of samples and several different assays, whether, seminal levels of cotinine, caffeine and other potential reproductive toxins, show any correlations with poor semen quality and/or spermatozoal damage.

Section C: Determination of Coenzyme Q_{10} in Seminal Fluid

C.6.1 Introduction

Coenzymes Q, or ubiquinones, represent a group of lipid soluble benzoquinones involved in the mitochondrial respiratory chain; specifically the oxidation of succinate or reduced nicotine adenine dinucleotide (Rauchova et al., 1995). The ubiquinone structure comprises a 2,3-dimethoxy-5-methylbenzoquinone nucleus with a variable length side-chain, containing one to twelve mono-unsaturated *trans*-isoprenoid units, as detailed in fig. C.6.1. Current nomenclature describes the compounds as coenzyme Q_n, where n equals the number of isoprenoid units, or ubiquinone *x*, where *x* designates the total number of carbon atoms in the side-chain and is a multiple of five. For example, coenzyme Q_{10} (CoQ_{10}), can otherwise be named ubiquinone 50, and contains ten isoprenoid units or fifty carbon atoms in its side-chain. Of the coenzyme Q family, only CoQ_{10} has been found in man (Okamoto et al., 1985; Battino et al., 1990).

Fig. C.6.1. Basic structure of the coenzymes Q. The compounds are based on a 2,3-dimethoxy-5-methylbenzoquinone nucleus with a side-chain containing one to twelve mono-unsaturated *trans*-isoprenoid units.



The cellular levels of CoQ_{10} determine the rate of respiration that occurs in mitochondria (Lenaz et al., 1985). The abundance of CoQ_{10} in tissues is dependent on both exogenous dietary intake and endogenous biosynthesis (Littarru et al., 1990). Diets deficient in CoQ_{10} produce a marked reduction in plasma levels of the compounds (Kishi et al., 1986). In addition, increased metabolic demand or destruction of lipids through oxidative damage may result in CoQ_{10} depletion, ultimately producing energy depletion (Littarru et al., 1990; Alleva et al., 1995).

Spermatozoal motility is highly dependent on a rich source of energy (Lewin and Lavon, 1997); therefore, reduced levels of CoQ_{10} might be expected to produce a reduction in cellular motility. Indeed, studies have demonstrated a positive correlation between seminal fluid concentrations of CoQ_{10} and sperm cell motility

(Mancini et al., 1994; Alleva et al., 1997). CoQ_{10} in seminal fluid has also been correlated with the sperm count in the ejaculate (Mancini et al., 1994; Alleva et al., 1997). In addition, seminal fluid levels of CoQ_{10} are significantly lower in oligo-, terato- and asthenozoospermic men compared to normozoospermic individuals (Mancini et al., 1994; Alleva et al., 1997).

In addition to its redox function, CoQ_{10} possesses potent antioxidant capacity and has the capacity to protect sperm from the generation of lipid peroxidation (Rauchova et al., 1995; Alleva et al., 1997). Since peroxidative processes are associated with the aetiology of infertility (Halliwell 1988; Aitken 1995; Suleiman et al., 1996), CoQ_{10} levels may be of further importance.

The antioxidant activity is lower in the seminal plasma of men who smoke compared to that of non-smokers (Fraga et al., 1996). As CoQ_{10} possesses strong antioxidant properties, levels of the compound may be lower in smokers. In addition, several reports have suggested that sperm from smokers possess a lower motility when compared to non-smokers (Pacifici et al., 1993; Vine et al., 1994; Sofikitis et al., 1995; Vine et al., 1996). This may be as a consequence of low CoQ_{10} levels and thus inadequate energy provision for the cells and/or reduced antioxidant capacity and increased oxidative damage. The purpose of the current study was to determine whether the levels of CoQ_{10} were different in sperm and/or seminal plasma obtained from normozoospermic smokers and non-smokers. We report that spermatozoal levels of CoQ_{10} were significantly associated with cell concentration, motility and morphology. However, no difference in spermatozoal or plasma levels were observed between smokers and non-smokers.

C.6.2 Methods

C.6.2.1 *Extraction of Coenzyme Q_{10} in Seminal Fluid using Liquid-Liquid Extraction*

Seminal fluid (1 ml) was centrifuged at 2000 x g for 10 min, to allow the separation of plasma from the spermatozoa. The supernatant comprising the seminal plasma or the cell pellet was resuspended in 1 ml sodium dodecyl sulphate (20 %, w/v in PBS), and 125 ng of CoQ_9, a ubiquinone that does not exist in man, added in ethanol to act as internal standard. After addition of 3 ml methanol : 1-propanol (95 : 5, v/v), the solution was vortexed for 30 s, and 4 ml of *n*-hexane added and the solution re-vortexed for 60 s. The aqueous and organic layers were separated by centrifugation (1000 x g for 10 min), and the upper organic phase collected. The aqueous phase was extracted again using the same procedure and the organic phases combined. The organic phase was dried under a stream of nitrogen and dissolved in 300 µl of ethanol, before analysis by HPLC.

C.6.2.2 *Extraction of Coenzyme Q_{10} in Seminal Fluid using Liquid-Liquid Extraction and Solid Phase Extraction Cartridges*

Extraction of CoQ_{10} in seminal fluid using liquid-liquid extraction and SPE cartridges was performed as described by Grossi et al. (1992). Seminal fluid (1.5 ml) was centrifuged at 2000 x g for 10 min, and the plasma separated from the spermatozoa. Seminal plasma (1 ml) or sperm (50 x 10^6) were resuspended in 1 ml sodium dodecyl sulphate (20 %, w/v in PBS; SDS), and 125 ng CoQ_9 in ethanol added to act as internal standard. After adding 3 ml of methanol : 1-propanol (95 : 5, v/v), the

solution was vortexed for 30 s, and 4 ml of *n*-hexane then added and the solution re-vortexed for 60 s. The aqueous and organic layers were separated by centrifugation at 1000 x g for 10 min, and the upper organic phase collected. The aqueous phase was extracted again using the same procedure and the organic phases combined. A 500 mg silica solid phase extraction (SPE) cartridge was conditioned with 2 ml *n*-hexane, before addition of the *n*-hexane biological extract. The cartridge was washed with 2 ml of *n*-hexane and dried under vacuum. Two 0.5 ml aliquots of methanol were then added to the cartridge and the eluent collected. The eluent was then purified on a C18 SPE cartridge, previously conditioned with 2 ml methanol and equilibrated with 2 ml water. After washing the cartridge with 1.5 ml methanol, the extracts were eluted with two 0.15 ml aliquots of 2-propanol, before analysis by HPLC.

C.6.2.3 *Chromatographic Analysis*

Cellular and plasma levels of CoQ_{10} were determined using HPLC with UV detection. The liquid chromatography system consisted of a Jasco PU-980 pump (Jasco UK, Great Dunmow, UK), a Hichrom 5 μm 150 x 4.6 mm C18 column equipped with a Hichrom guard column (5 μm, 10 x 4.6 mm; Hichrom Limited, Reading, UK), and a Jasco UV-975 detector set at 275 nm. The mobile phase consisted of ethanol : methanol (60 : 40 v/v) that was filtered through a 0.45 μm filter under vacuum and degassed using helium before use. A 50 μl injection volume was used for each extract. All separations were carried out at ambient room temperature, using a flow rate of 1 ml/min.

C.6.3 Results

C.6.3.1 *Profile of Semen Obtained from Smokers and Non-smokers used in the Study*

Semen obtained from men who smoked for use in the study possessed significantly lower sperm cell concentrations and reduced motility when compared to semen from non-smokers ($P < 0.05$; table C.6.1).

Table C.6.1. Profile of semen obtained from smokers and non-smokers used in the determination of coenzyme Q_{10} levels. Values are mean ± SD.

Donor group	Volume (ml)	Sperm count (x 10^6/ml)	Motility (% forward progression)	Normal forms (%)
Smoker (n = 15)	2.5 ± 1.1	71.13 ± 31.03	66.93 ± 10.65*	57.73 ± 17.11
Non-smoker (n = 15)	2.7 ± 0.7	86.73 ± 30.48	75.87 ± 12.23	55.60 ± 16.08

* Represents a significant difference from the semen obtained from non-smokers ($P < 0.05$).

C.6.3.2 *Measurement of Coenzyme Q_{10} in Seminal Fluid*

Dissociation of CoQ_{10} from lipids and proteins using sodium dodecyl sulphate and methanol, followed by liquid extraction using *n*-hexane, did not provide adequate chromatographic resolution of CoQ_{10} or CoQ_9, and therefore was unsatisfactory for use in the analysis (fig. C.6.2 - C.6.3).

Fig. C.6.2. Typical chromatogram of a spermatozoal extract executed as described under materials and methods, section C.6.2, using liquid-liquid extraction with *n*-hexane, to allow the measurement of coenzyme Q_{10}. The mobile phase consisting of ethanol : methanol (60 : 40, v/v), produced retention times of 12.20 min for coenzyme Q_{10} and 8.10 min for the internal standard, coenzyme Q_9.

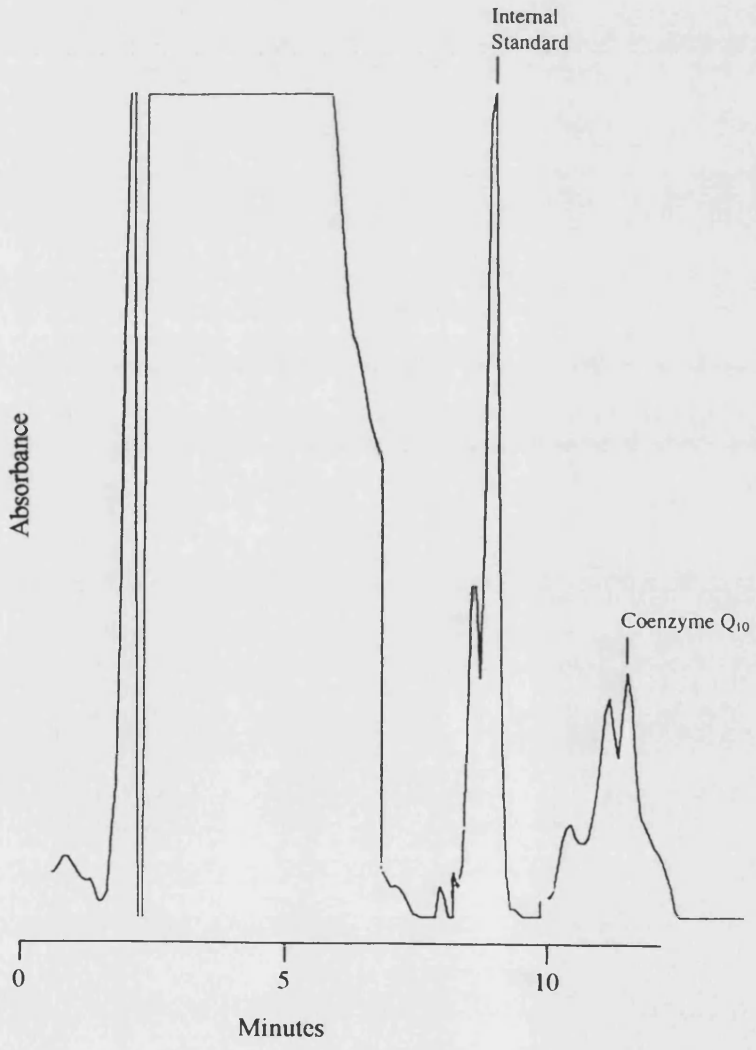
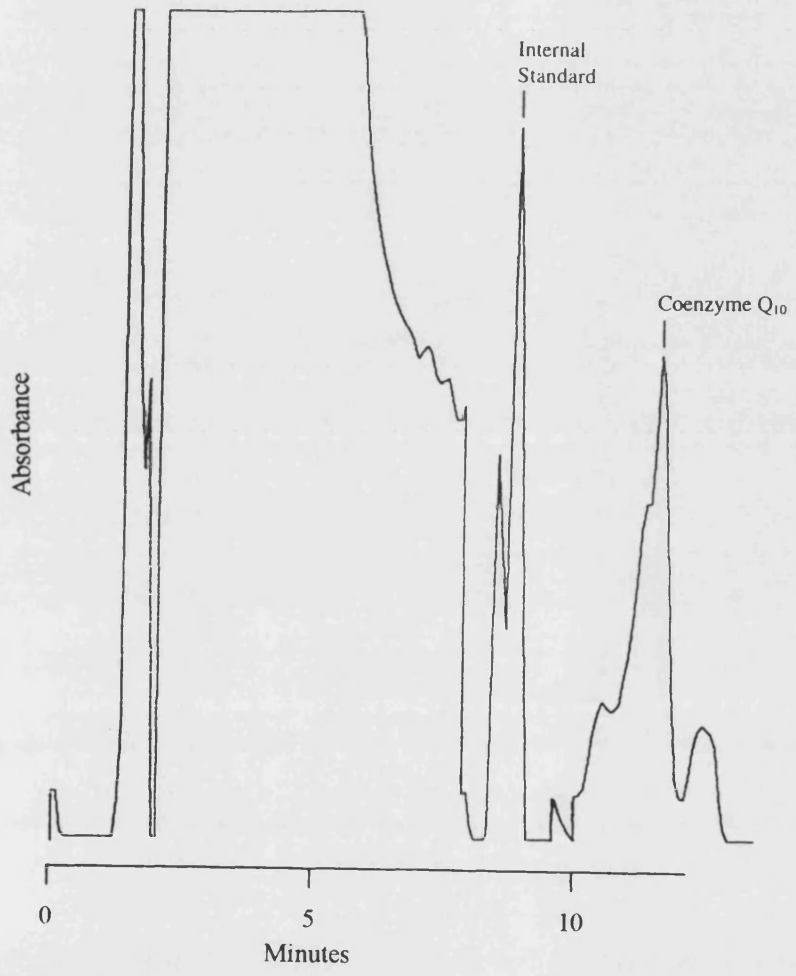


Fig. C.6.3. Typical chromatogram of a seminal plasma extract executed as described under materials and methods, section C.6.2, using liquid-liquid extraction with *n*-hexane, to allow the measurement of coenzyme Q_{10}. The retention times of coenzyme Q_{10} and coenzyme Q_9 were the same as those of the spermatozoal extract (fig. C.6.2).



Seminal fluid *n*-hexane extracts followed by SPE concentration and clean-up steps produced a cleaner chromatogram when compared to liquid extraction alone, and produced adequate resolution of both CoQ_9 and CoQ_{10} (fig. C.6.5 - C.6.6). The inclusion of the SPE steps after liquid extraction was therefore implemented for the subsequent analysis.

When SPE was implemented following liquid extraction, CoQ_{10} recovery was high (93.73 ± 2.72 % for seminal plasma and 89.26 ± 2.18 % for spermatozoa). Fig. C.6.4 displays the calibration curve produced following extraction of CoQ_{10} from seminal plasma and sperm using *n*-hexane followed by SPE clean-up and concentration steps. The analysis was linear up to a concentration of 250 ng/ml CoQ_{10} (r = 0.992).

Fig. C.6.4. Calibration curve for the analysis of CoQ_{10}, following *n*-hexane liquid-liquid extraction and solid phase extraction cartridge clean-up and concentration steps.

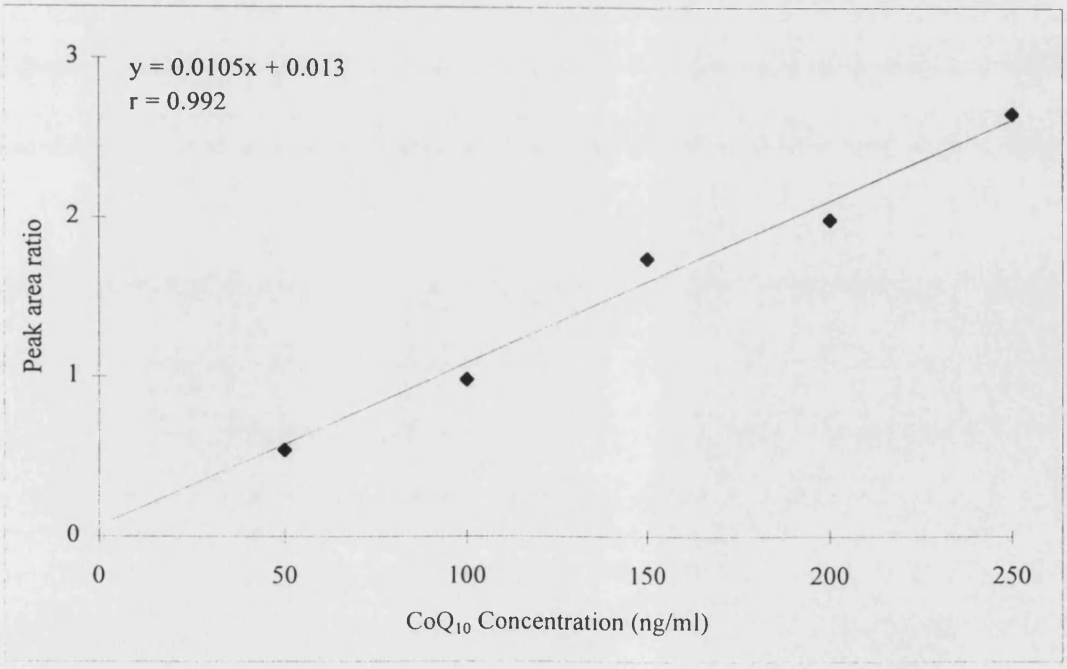


Fig. C.6.5. Typical chromatogram of a spermatozoal extract executed as described under materials and methods, section C.6.2, using liquid-liquid extraction with *n*-hexane followed by concentration and clean-up using solid phase extraction (SPE) cartridges, to allow the measurement of coenzyme Q_{10}. The employment of SPE cartridges after liquid extraction using *n*-hexane resulted in a far cleaner chromatogram compared to liquid extraction alone.

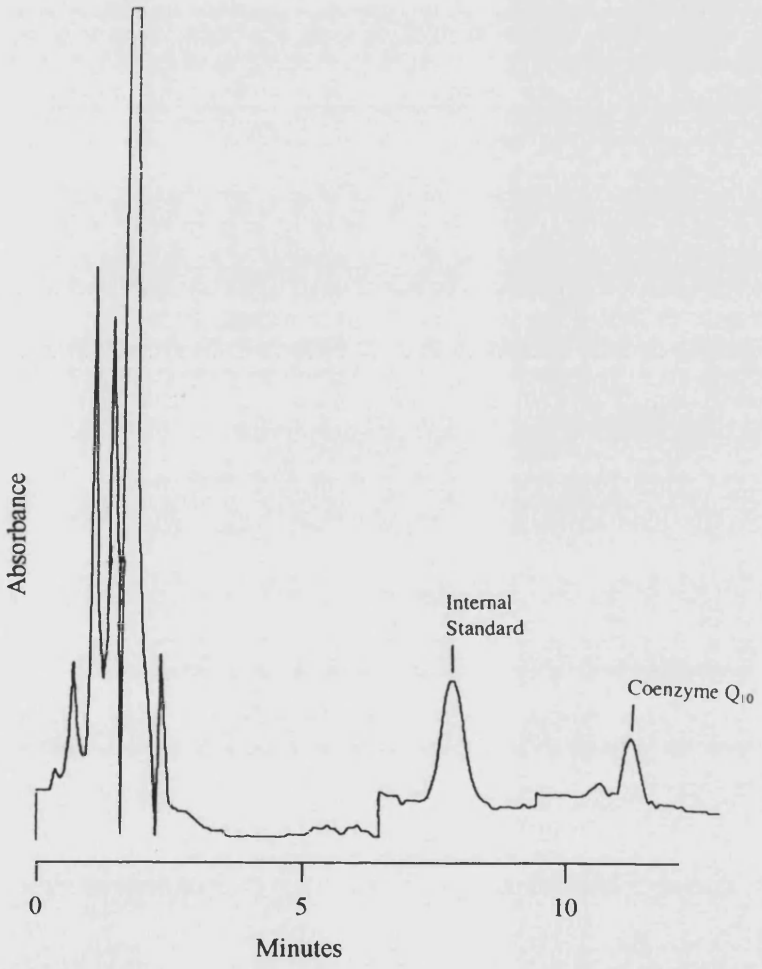
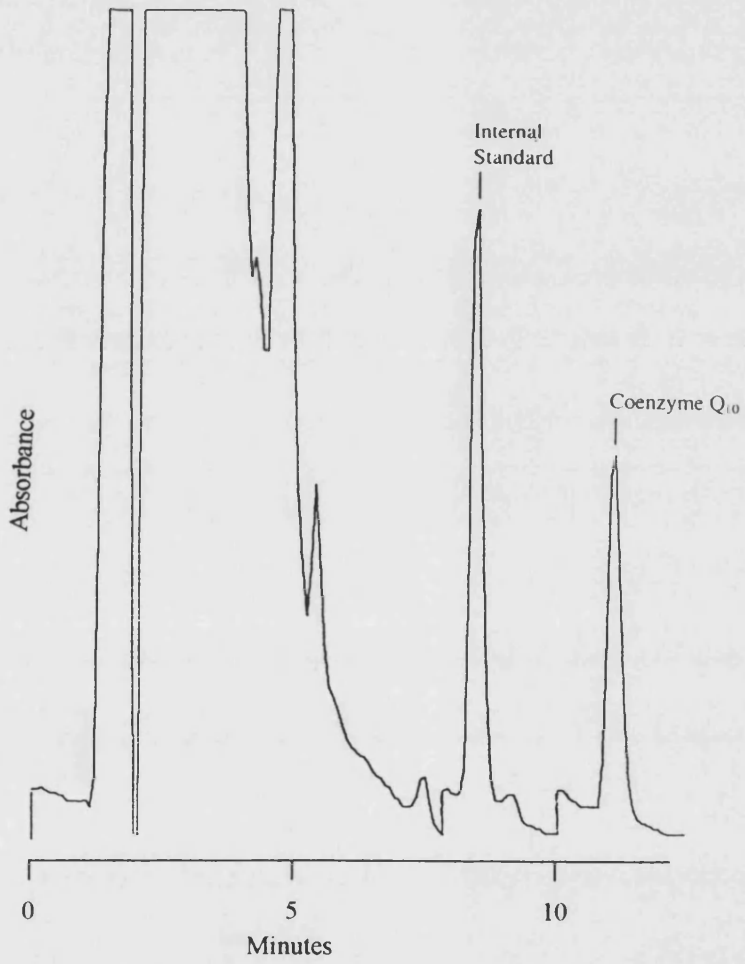


Fig. C.6.6. Typical chromatogram of a seminal plasma extract executed as described under materials and methods, section C.6.2, using liquid-liquid extraction with *n*-hexane followed by concentration and clean-up using solid phase extraction (SPE) cartridges, to allow the measurement of coenzyme Q_{10}. The employment of SPE cartridges after liquid extraction using *n*-hexane resulted in a far cleaner chromatogram compared to liquid extraction alone.



The mean amount of CoQ_{10} in spermatozoa was found to be 0.54 ± 0.27 ng/10^6 sperm, and in seminal plasma 27.65 ± 2.09 ng/ml. A positive correlation was observed between spermatozoal CoQ_{10} levels and cell number and motility in both smokers and non-smokers (fig. C.6.7, C.6.9; $P<0.05$). A positive correlation was also observed between CoQ_{10} levels and normal cell morphology in the non-smokers data group (fig. C.6.11; $P<0.05$). No correlation was observed between seminal plasma levels of CoQ_{10} and any semen parameter ($P>0.05$).

Levels of CoQ_{10} in both spermatozoa and seminal plasma did not differ between smokers and non-smokers ($P>0.05$, *t* test), as can be seen in table C.6.2.

Table C.6.2. Levels of CoQ_{10} in the spermatozoa and seminal plasma of smokers and non-smokers.

Group	CoQ_{10} in spermatozoa	CoQ_{10} in seminal plasma
Non-smokers	0.59 ± 0.28 ng/10^6 sperm	27.24 ± 2.11 ng/ml
Smokers	0.50 ± 0.24 ng/10^6 sperm	28.06 ± 2.05 ng/ml

No difference was observed in the amount of CoQ_{10} between smokers and non-smokers in either the spermatozoal or seminal plasma fraction ($P>0.05$).

Fig. C.6.7. Correlation of CoQ_{10} levels in spermatozoa with sperm count in normozoospermic donors, who were classified as non-smokers (fig. C.6.7a) or smokers (fig. C.6.7b). The lines indicate the trend produced by linear regression.

Fig. C.6.7a

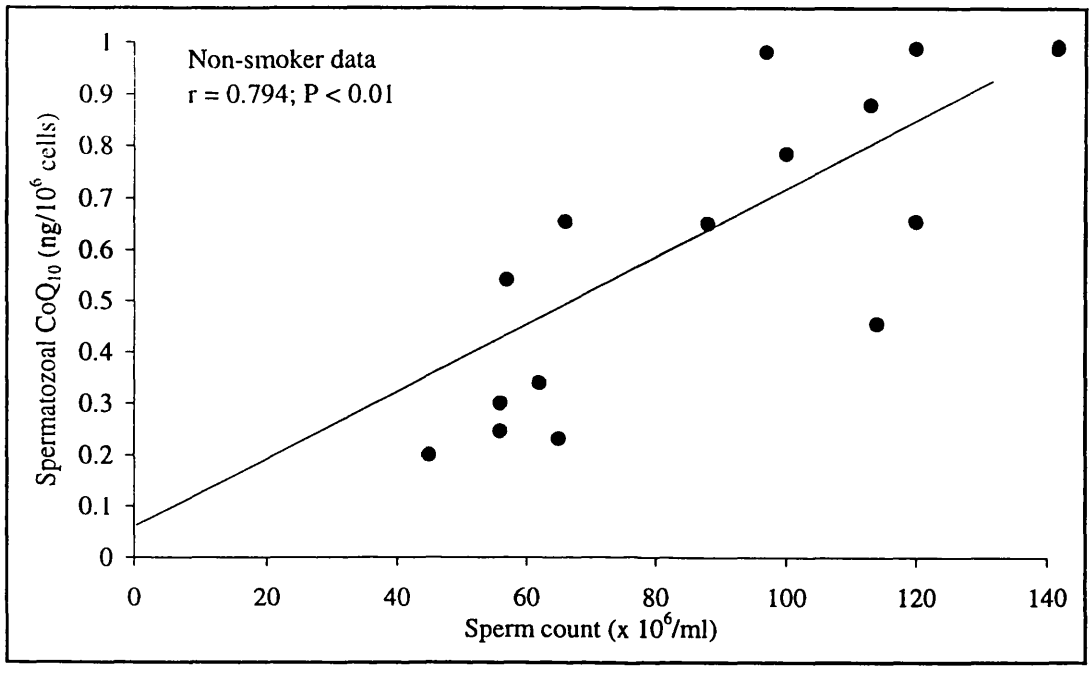


Fig. C.6.7b

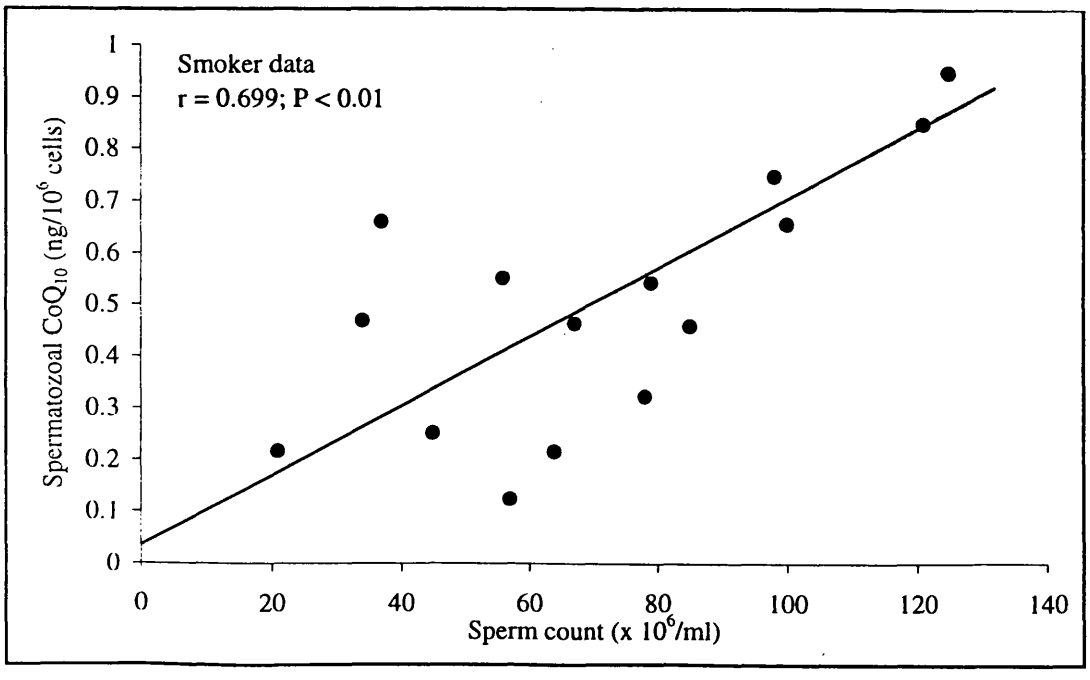


Fig. C.6.8. Correlation of CoQ_{10} levels in seminal plasma with sperm count in normozoospermic donors, who were classified as non-smokers (fig. C.6.8a) or smokers (fig. C.6.8b). The lines indicate the trend produced by linear regression.

Fig. C.6.8a

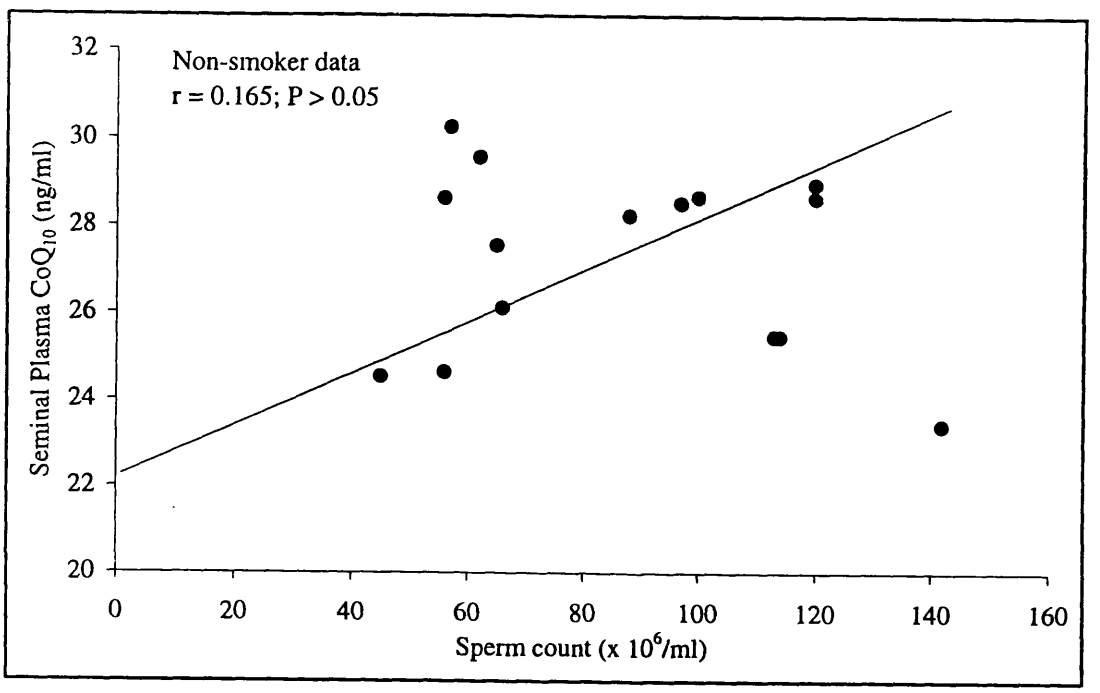


Fig. C.6.8b

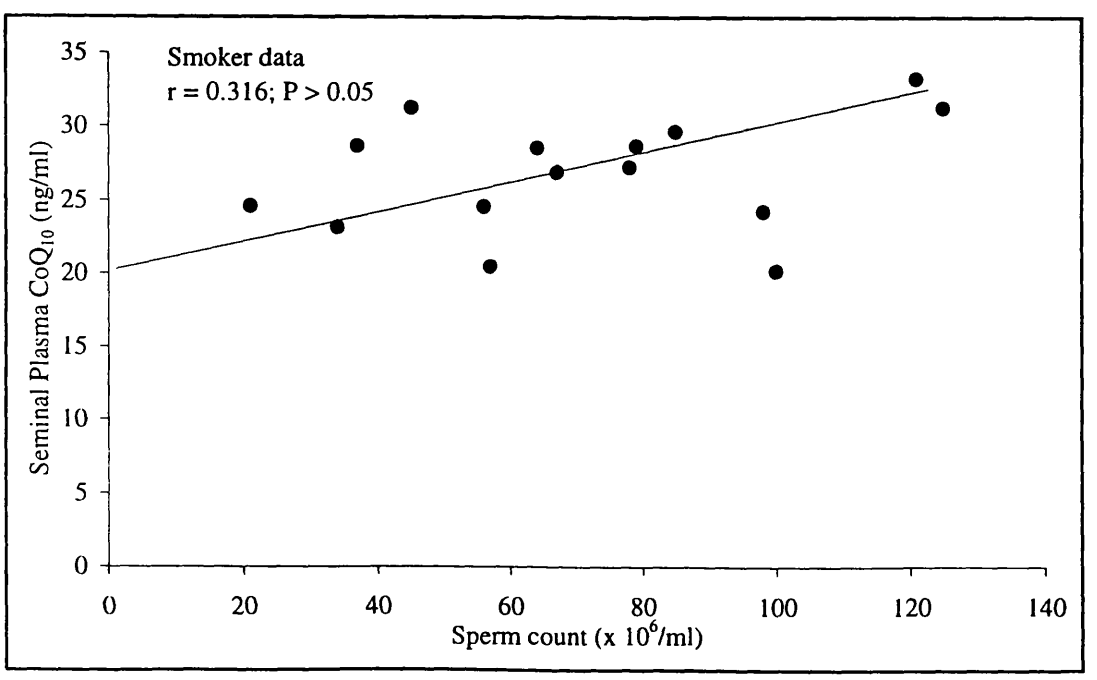


Fig. C.6.9. Correlation of CoQ_{10} levels in spermatozoa with sperm motility in normozoospermic donors, who were classified as non-smokers (fig. C.6.9a) or smokers (fig. C.6.9b). The lines indicate the trend produced by linear regression.

Fig. C.6.9a

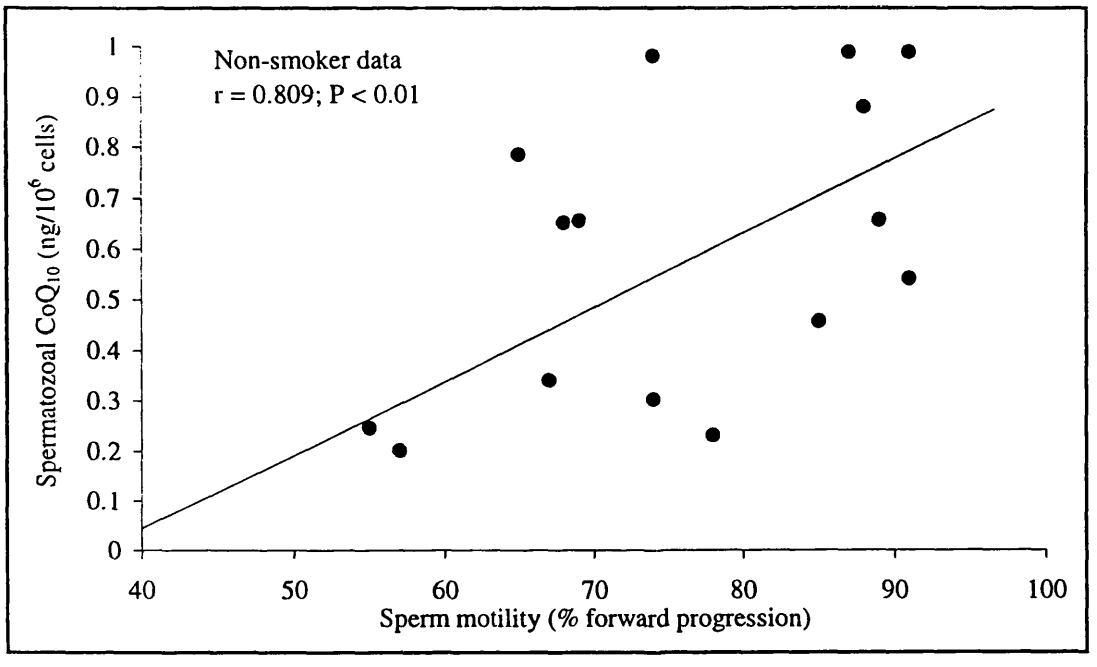


Fig. C.6.9b

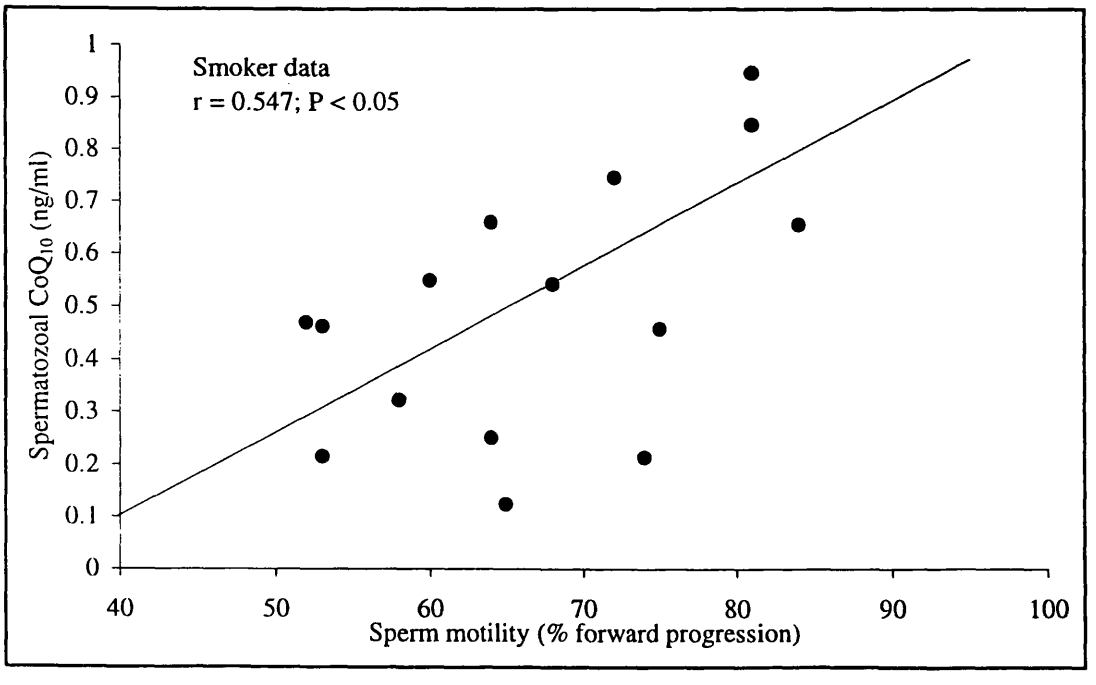


Fig. C.6.10. Correlation of CoQ_{10} levels in seminal plasma with sperm motility in normozoospermic donors, who were classified as non-smokers (fig. C.6.10a) or smokers (fig. C.6.10b). The lines indicate the trend produced by linear regression.

Fig. C.6.10a

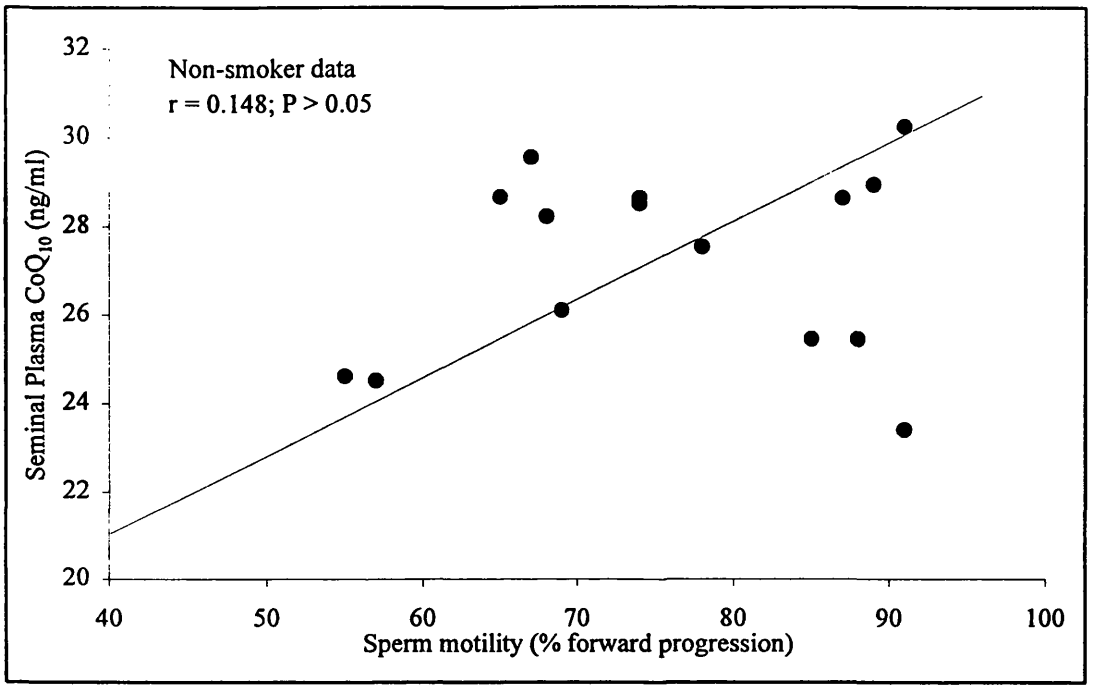


Fig. C.6.10b

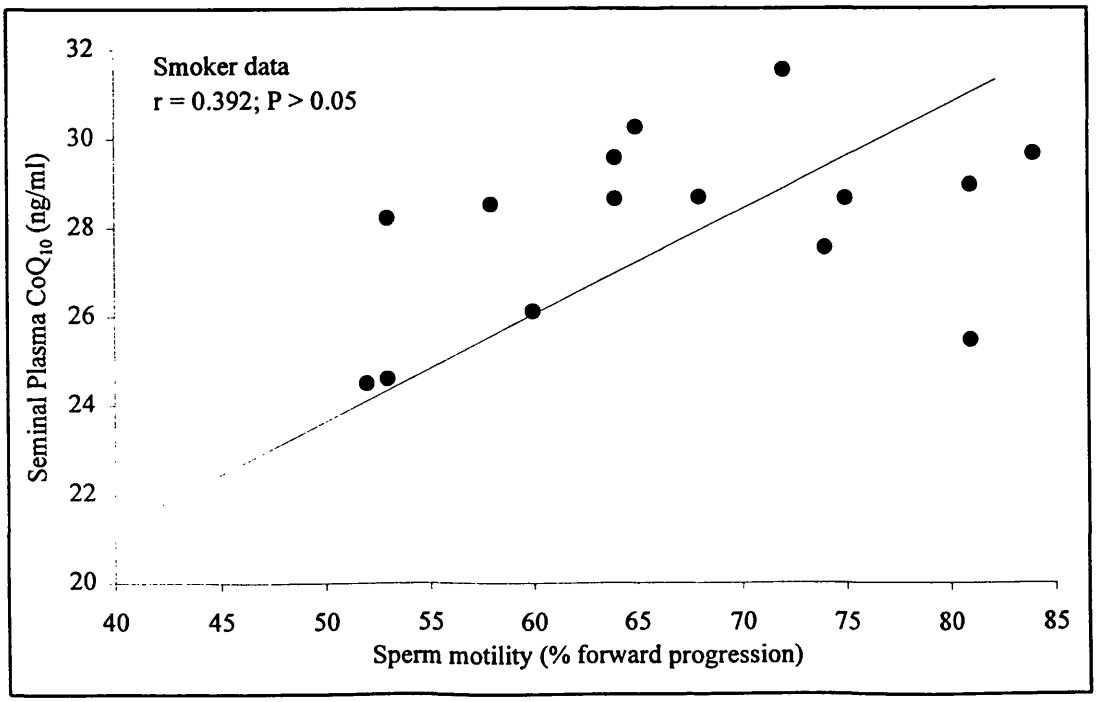


Fig. C.6.11. Correlation of CoQ_{10} levels in spermatozoa with sperm morphology in normozoospermic donors, who were classified as non-smokers (fig. C.6.11a) or smokers (fig. C.6.11b). The lines indicate the trend produced by linear regression.

Fig. C.6.11a

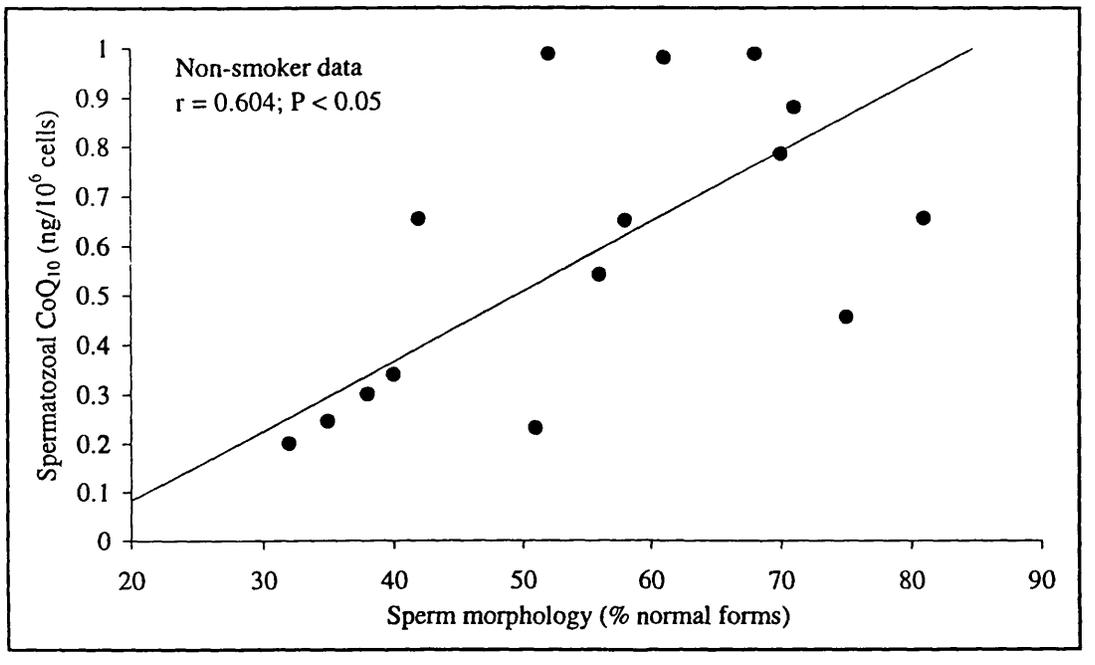


Fig. C.6.11b

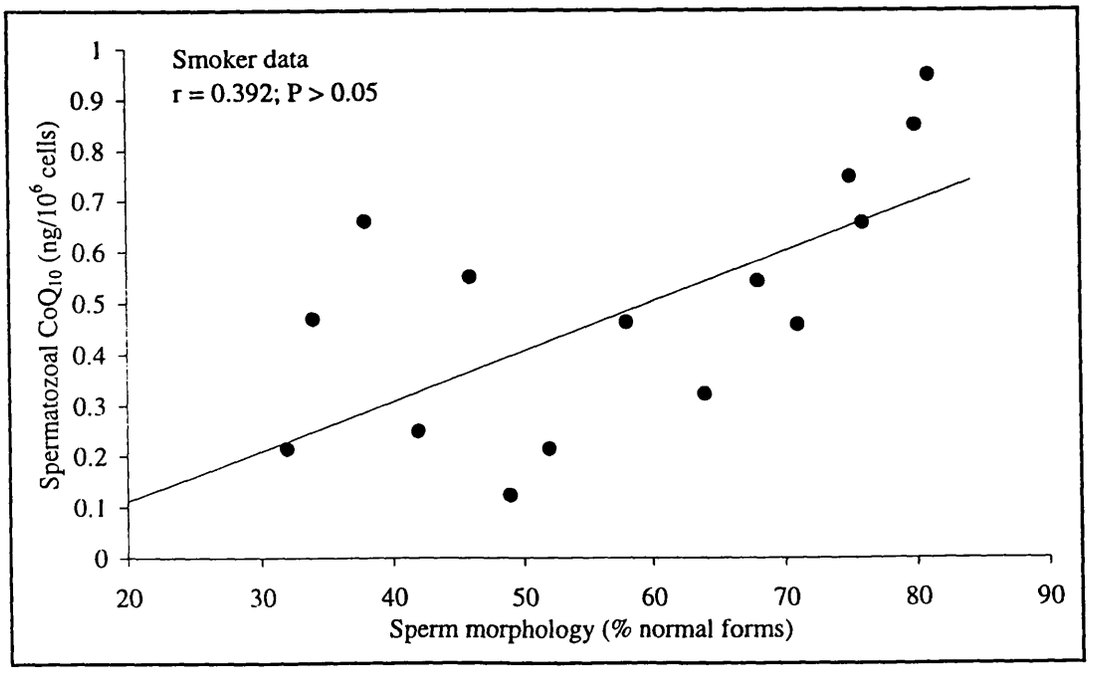


Fig. C.6.12. Correlation of CoQ_{10} levels in seminal plasma with sperm morphology in normozoospermic donors, who were classified as non-smokers (fig. C.6.12a) or smokers (fig. C.6.12b). The lines indicate the trend produced by linear regression.

Fig. C.6.12a

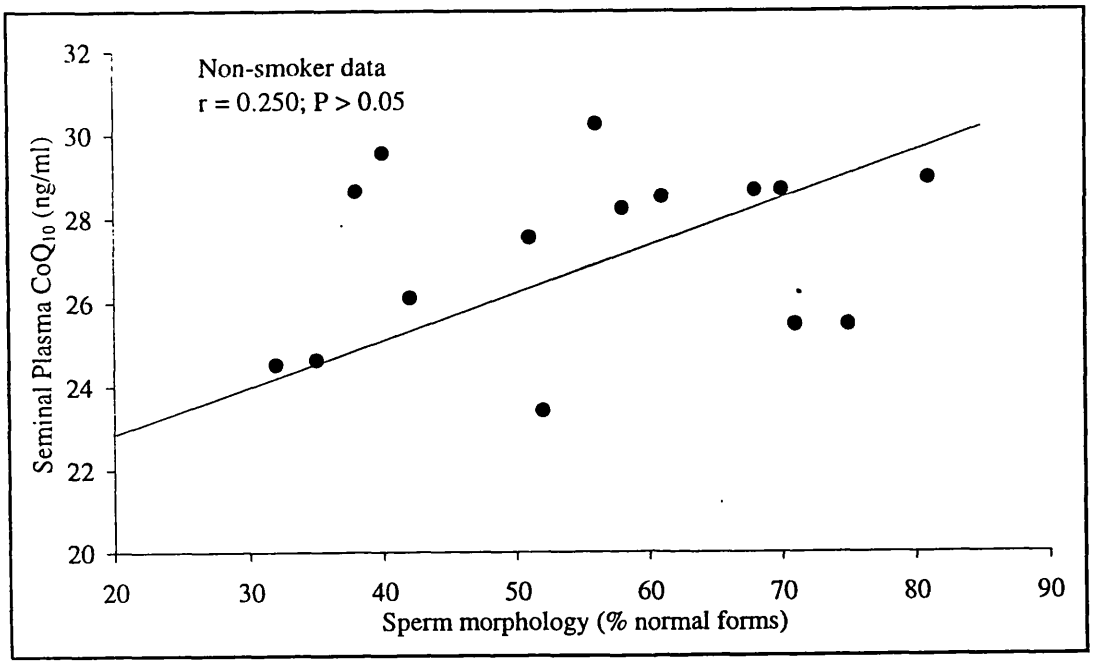
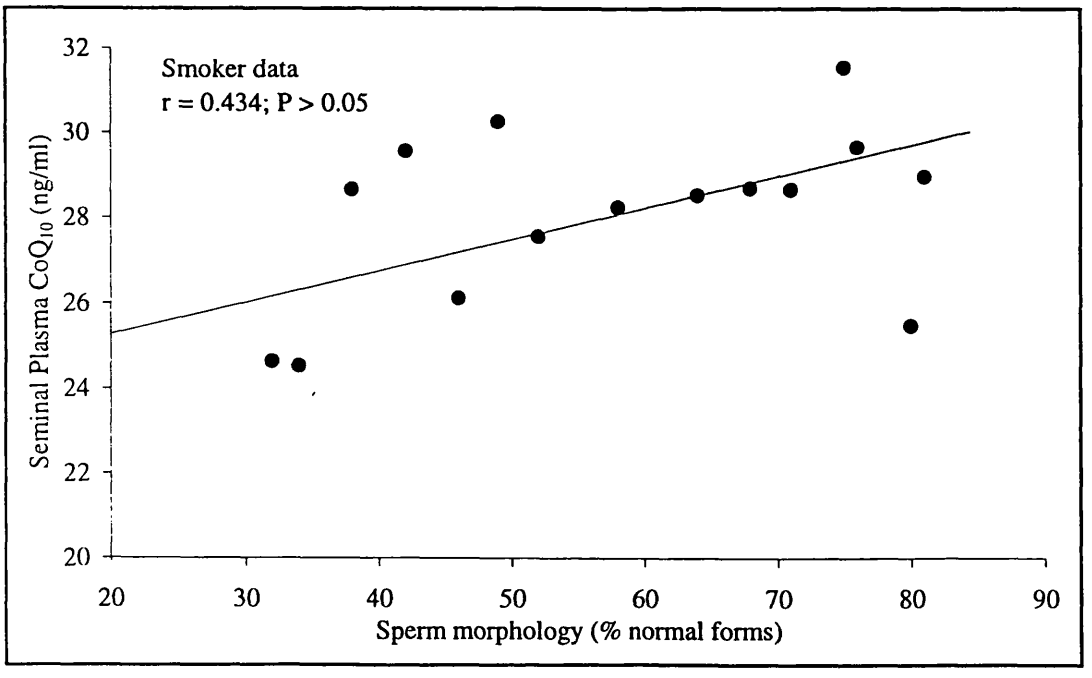


Fig. C.6.12b



C.6.4 Discussion

Cigarette smoking is associated with oxidative damage and reduced levels of antioxidants in semen (Fraga et al., 1996; Shen et al., 1997). In addition, smoking has been negatively correlated with spermatozoal motility (Pacifici et al., 1993; Sofikitis et al., 1995; Vine et al., 1996). This study indicates that CoQ_{10} levels are not significantly different in the seminal plasma or spermatozoa of smokers compared to non-smokers. Thus, the impaired motility that has been observed in sperm from smokers in several reports, including this study, does not appear to be as a consequence of reduced CoQ_{10} concentrations and subsequent lowered energy availability and/or reduced protection against oxidative attack. However, the significant correlations that were found between sperm count, motility and morphology and CoQ_{10} levels in spermatozoa, does indicate an important role of the compound in these parameters.

In conclusion, the cellular basis for the potential reduction of spermatozoal motility in cigarette smokers requires further consideration. Reduced levels of antioxidants, such as α-tocopherol, have been associated with a decrease in sperm cell motility (Geva et al., 1996; Suleiman et al., 1996). The lower antioxidant capacity demonstrated in smokers seminal plasma (Fraga et al., 1996; section 3.3.3) may account therefore for the lowered spermatozoal motility observed previously (Pacifici et al., 1993; Sofikitis et al., 1995; Vine et al., 1996).

7. Conclusions and Future Work

7.1 Summary of Results

The purpose of the present study was to determine whether smoking and/or depleted antioxidant levels causes spermatozoal damage, that may result in reduced fertility and offspring pathology. The study has determined that male cigarette smoking results in adverse effects on sperm. It was found that cigarette smoking produces damage to the chromatin of sperm. Specifically, greater susceptibility to acid-induced denaturation of DNA, increased levels of DNA strand breaks and higher frequency of YY1 aneuploidy was witnessed. Additionally, smoking was associated with lower seminal plasma total antioxidant activity. Smoking was not, however, associated with levels of spermatozoal or seminal plasma lipid peroxidation, according to the measurement of TBARS.

The protection afforded by antioxidants to spermatozoa was also investigated. Seminal plasma was found to reduce the formation of DNA strand breaks and lipid peroxidation after addition to an incubation media containing H_2O_2, $FeSO_4$ and ADP. The amount of seminal plasma present was also significantly negatively correlated with the formation of lipid peroxides. The epididymis provides optimum conditions for the storage and maturation of sperm (Hinton et al., 1995). The antioxidant capacity of the epididymal fluid, therefore, may be of importance in maintaining the integrity of spermatozoa whilst stored at this site. The epididymal antioxidant capacity was investigated by comparing the seminal fluid of normozoospermic and vasectomized men. The results indicate that the epididymis possesses region-specific

antioxidant activity which may protect sperm from oxidative attack whilst stored at this site. In addition, the work has demonstrated that extra-epididymal storage of sperm is associated with spermatozoal damage, namely increased levels of chromatin damage, namely reduced protamine disulphide crosslinking, deficient chromatin condensation, and increased susceptibility to acid-induced denaturation, combined with a loss of cell motility.

CoQ_{10}, a component of the respiratory chain and potent antioxidant, was quantified in spermatozoa and seminal plasma obtained from either smokers or non-smokers. It was hypothesised that levels of seminal CoQ_{10} might be lower in smokers' semen compared to non-smokers, due to the lower total antioxidant capacity that was previously determined in the smoker's group. Reduced CoQ_{10} levels may then explain, at least in part, the reduced spermatozoal motility observed in smokers compared to non-smokers due to energy deprivation. However, the amount of CoQ_{10} in either the spermatozoa or seminal plasma was not different in specimens obtained from smokers and non-smokers. Spermatozoal levels of CoQ_{10} were, however, positively correlated with sperm count, motility and normal morphology.

The extraction of large volumes of semen using supercritical fluid followed by GC-MS analysis was performed in order to assess whether semen contains chemicals capable of causing damage to cells of the male and female reproductive tract. The extracts provided identification of nicotine and cotinine according to their mass spectra and retention times. Neither compound was detectable in the semen of non-smokers, concurring with the data obtained following supercritical extraction of small volumes of seminal fluid and subsequent chromatographic analysis using HPLC with

UV detection. Caffeine was also detected in the seminal fluid of both smokers and non-smokers. The aromatic hydrocarbon, 1,2 benzenedicarboxylic acid, was also present in the semen of both smokers and non-smokers.

Supercritical fluid technology was also successfully applied to the extraction of caffeine and cotinine from seminal fluid. Specimens obtained from smokers contained detectable levels of cotinine; samples donated by non-smokers did not contain detectable amounts of cotinine. This data also validates the results gained during the study, for only semen that was obtained from men who declared to be smokers contained cotinine. Semen donated from individuals who reported that they were non-smokers, did not contain cotinine.

7.2 Discussion of Results

Cigarette smoking causes severe oxidative stress (Halliwell and Gutteridge, 1999). This study has demonstrated a reduction in seminal plasma antioxidant levels in the semen of men who smoke compared to those who do not. Lower antioxidant activity and seminal plasma oxidative stress have been associated with a reduction in sperm motility (Kobayashi et al., 1991; Aitken 1995; Geva et al., 1996; Suleiman et al., 1996). A reduction in spermatozoal motility in smokers' semen has been found in this and several other studies (Pacifici et al., 1993; Vine et al., 1994; Sofikitis et al., 1995; Vine et al., 1996); the reduction may be as a direct consequence of the reduced abundance of antioxidants and subsequent higher levels of cellular oxidative stress.

Low levels of seminal plasma antioxidants have also been associated with spermatozoal DNA damage (Fraga et al., 1991). Therefore, reduced seminal plasma

antioxidant capacity, as demonstrated in men who smoked compared to those who did not, may indirectly lead to greater generation of DNA damage and possible mutagenesis.

Exposure of sperm to ROS can result in DNA damage, which is reduced by the presence of antioxidants and seminal plasma. Antioxidant levels in semen should, therefore, be maintained during the storage and manipulation of cells for in vitro fertility work, in order to reduce damage to the sperm.

The epididymis is known to act as the main site for the storage of sperm, whilst enabling the correct maturation of sperm (Hinton et al., 1995). It would seem from the results in the current study, that the epididymis also prevents the reduction of nuclear disulphide bonds and production of an abnormal chromatin structure. This may be partly due to the presence of antioxidants in the epididymal lumen itself, that was also demonstrated.

Epidemiological studies have implicated an association between paternal cigarette smoking and offspring pathology (Zhang et al., 1992; Sorahan et al., 1995; 1997a; 1997b), in addition to an increased risk of cervical cancer in the partners of male smokers (Bosch et al., 1996). The presence of nicotine and cotinine in the seminal fluid of smokers has been confirmed previously (Pacifici et al., 1993). Both compounds are precursors of the mutagenic and carcinogenic nitrosamines. Although no known human mutagen or carcinogen has yet been discovered in semen, further work may positively identify such a compound. This would have important implications, not only to the production of spermatozoal DNA damage and mutations,

but also to the possible association with male cigarette smoking and cervical cancer of their partners (Bosch et al., 1996).

The compound 1,2 benzenedicarboxylic acid, was detected using GC-MS after the extraction of semen with SFE. The aromatic nature of the compound hints towards its possible mutagenicity, akin to the mutagenicity and carcinogenicity of other aromatic compounds, such as PAH. It should be noted, however, that 1,2 benzenedicarboxylic acid is used in the synthesis of plastics (Standard ChemStation software 1996); thus, the notion that the compound is a component of the plastic containers used for the storage of semen, and does not actually reside in the seminal fluid itself, should be borne in mind.

7.3 Future Work

The greater damage observed in the chromatin of sperm from smokers compared to non-smokers requires future consideration. Potential correlations with DNA damage, frequencies of sperm aneuploidy, seminal plasma antioxidants, and cigarette consumption according to plasma cotinine concentration, for example, should be evaluated. The frequencies of aneuploidy should be determined for chromosomes not examined in the current study in a broad cross section of men, such as those of low fertility, smokers or heavy consumers of caffeine. Additionally, future studies should attempt to control in each experiment variables that exist in the semen donors. For example, donor age, use of pharmaceuticals, and cigarette, caffeine and alcohol consumption.

We have indicated an association of smoking with sperm mutations, namely YY1 aneuploidy. Future studies should assess whether smoking causes further germline mutations, which may lead to birth defects and genetic disease in offspring. Certain epidemiological studies indicate that this may be the case (Zhang et al., 1992; Sorahan et al., 1995; 1997a; 1997b). In addition, modern analytical techniques should be used to determine whether mutagenic and carcinogenic compounds and/or their metabolites, such as TSNA and PAH, are present in seminal plasma. The measurement of DNA adducts of such chemicals should also be rigorously undertaken, in order to provide estimations of the possible incidence of germline mutations. Relationships with smoking status and antioxidant concentrations, if present, should be established.

Regarding the protective effect of seminal plasma on spermatozoa, future work should determine whether the protection afforded by seminal plasma is correlated with its antioxidant properties. In addition, whether certain populations, such as smokers and men of low fertility, individuals that possess lower plasma antioxidant levels and higher levels of DNA damage, are more susceptible to the effects of oxidative damage should be addressed.

Associations with smoking and male fertility requires further investigation. Several studies have correlated cigarette consumption with a reduction in sperm count and motility and an increase in the number of abnormal cells (Pacifici et al., 1993; Vine et al., 1994; Sofikitis et al., 1995; Vine et al., 1996). Data should be collected to assess whether smokers have greater difficulty conceiving than non-smokers, both during in vitro fertilisation work and in vivo. Research should be performed to determine

whether men who are of low fertility and/or low in plasma antioxidants, are able to improve their chances of parenthood through cessation of smoking or by increasing their plasma antioxidant levels, for example, by raising dietary intake of such compounds.

Although we feel that the conclusions reached in the present study are justified, an important qualification is emphasised. Our samples of normozoospermic semen were obtained from the male partners of infertile couples and it remains largely unknown to what extent these are truly normal; i.e. comparable to that in normozoospermic men of proven fertility. It is possible, for example, that samples from men who have conceived and are, therefore, of proven fertility, would provide differing results as to those found in this study. For instance, sperm obtained from men of proven fertility might show even more extreme differences than that observed when 'normozoospermic' sperm were compared with spermatozoa from men post-vasectomy. It will be of importance in future studies to test whether the results found in this study remain true in samples obtained from men of proven fertility.

In conclusion, this study has discovered several novel findings that may have important implications for male reproductive toxicology. The reduction in human semen quality, that appears to be occurring currently and through the last century (Carlsen et al., 1992), has prompted the need for a more extensive knowledge of the male reproductive system. In particular, further information regarding compounds that are capable of being beneficial or detrimental to male fertility is required. Additionally, more sensitive and specific laboratory assays and increased sample sizes should be used to establish whether certain factors, such as oxidative stress and

toxic chemicals, induce sperm mutations which may result in birth defects and genetic disease in offspring. This would then allow suitable recommendations and safety precautions to be put into place; so as to protect human populations. A thorough evaluation of potential adverse effects on male reproduction should be included in drug developmental studies. It is hoped that this study will add significantly to the information currently available in this area, and lead to more effective methods for improving fertility and reducing morbidity in the human population.

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